PHYSIOLOGICAL STUDIES ON CULTIVAR-SPECIFIC RESISTANCE OF TOMATO PLANTS TO *CLADOSPORIUM FULVUM*

Promotor: dr.ir. J. Dekker, hoogleraar in de fytopathologie

P.J.G.M. DE WIT

PHYSIOLOGICAL STUDIES ON CULTIVAR-SPECIFIC RESISTANCE OF TOMATO PLANTS TO *CLADOSPORIUM FULVUM*

Proefschrift
ter verkrijging van de graad van
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aan Els,
Christiaan en Matthieu

STELLINGEN

I

Accumulatie van fytoalexinen door elicitors die voorkomen in cultuurfiltraten en celwanden van *Cladosporium fulvum* is noch fysio- noch cultivar- noch waardplant-specifiek.

Dit proefschrift

II

Indien aan het voorkomen van polyacetylenen als taxonomisch kenmerk veel waarde gehecht wordt bij de indeling van het plantenrijk, dan zijn de Solanaceeën nauwer verwant aan de Composieten en Umbelliferen dan meestal wordt aangenomen.

Dahlgren, R. (1977). *Plant Syst. Evol.* Suppl. 1, 253-283
Dit proefschrift

III

De argumenten die Ciferri en Ellis hanteren om *Cladosporium fulvum* over te brengen naar een nieuw geslacht *Fulvia* zijn niet overtuigend. Het bezigen van de tot nu toe gebruikte naam is te prefereren.

Ciferri, R. (1954). *Atti Ist. bot. Univ. Lab. crittogam. Pavia* 10, 245-246
Ellis, M.B. (1976). In *More Dematiaceous Hyphomycetes*. Commonwealth Agricultural Bureaux, Slough.

IV

Er zijn aanwijzigingen dat de opvatting van vele fytopathologen, dat bij cultivar-specifieke resistentie de incompatibele interactie specifiek is, waarschijnlijk onjuist is.

Heath, M.C. (1980). *Phytopathology* 70, 356-360
Doke, N. & Tomiyama, K. (1980). *Physiol. Plant Pathol.* 16, 177-186

V

De algemeen aanvaarde theorie dat parasitaire schimmels afstammen van saprofytische voorouders is aan bedenkingen onderhevig.

Lewis, D.H. (1973). *Biol. Rev.* 48, 261-278
Cooke, R.C. & Whipps, J.M. (1980). *Biol. Rev.* 55, 341-362

VI

Het is onwaarschijnlijk dat het systemisch fungicide efosiet-aluminium (Aliette) zijn werkzaamheid ontleent aan verhoogde synthese van fytoalexinen.

Hai, V.T., Bompeix, G. & Ravisé, A. (1979). *C.R. Acad. Sci. Paris* 288, 1171-1174

VII

Het model voor de organisatie en strekkingsgroei van de primaire celwand van dicotyle planten, opgesteld door Albersheim en medewerkers, is voor kritiek vatbaar.

Keegstra, K., Talmadge, K.W., Bauer, W.D. & Albersheim, P. (1973). *Plant Physiol.* 51, 188-196
Monro, J.A., Penny, D. & Bailey, R.W. (1976). *Phytochemistry* 15, 1193-1198

VIII

Vele plantaardige polyacetylenen ontlenen hun biologische werking mogelijk aan hun fotodynamische activiteit.

Towers, G.H.N. (1980). *Prog. in Phytochem.* 6, 183-222

IX

De biotoetsen die tot nu toe gebruikt worden in onderzoek naar de moleculaire basis van cultivar-specifieke resistentie, zoals de inductie van necrose, accumulatie van fytoalexinen, lignificatie en vorming van callose, zijn waarschijnlijk ongeschikt voor het opsporen van "specificiteitsfactoren".

X

Veranderingen in antigene eigenschappen van het celwand oppervlak zouden voor parasitaire schimmels een mechanisme kunnen zijn om aan de natuurlijke afweer van hun waardplant te ontsnappen.

XI

De verhouding tussen ontwikkelde en ontwikkelingslanden lijkt op die tussen de hoofdpersoon en zijn slachtoffer in het gedicht van L. Tolstoj:
"Ik zit op iemands rug, doe hem naar adem snakken en laat mij door hem dragen. En toch blijf ik tegen mijzelf en anderen zeggen dat ik medelijden met hem heb en zijn last zou willen verlichten, door te doen wat ik kan, behalve door van zijn rug af te gaan".

XII

De uitvinder van het schaakspel is vrijwel zeker een voorstander van de emancipatie van de vrouw geweest, daar de koningin oppermachtig is en de koning schaakmat gezet dient te worden.

P.J.G.M. de Wit.
Wageningen, 24 april 1981.

VOORWOORD

Bij de afronding van dit proefschrift zou ik graag allen die aan de totstandkoming ervan hebben bijgedragen willen bedanken.

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Academic Press and the Netherlands Society of Plant Pathology have given permission to reprint the articles incorporated in this thesis.

Tenslotte wil ik allen die hierboven niet genoemd zijn, maar toch mijn onderzoek met belangstelling gevolgd hebben, hartelijk danken.

CONTENTS

INTRODUCTION

General.

Plants at all stages of their life cycle are exposed to many potentially parasitic microorganisms. Under favourable conditions of moisture and temperature these microorganisms may attempt to infect plants. These attempts, however, often fail and most plants remain healthy. Resistance of plants to parasites appears to be the rule and susceptibility the exception. Usually, parasitic microorganisms have a highly limited host range.

The term specificity in plant-parasite interactions is used to refer to the ability of an infectuous agent to cause disease in particular plants, but not in others. Many gradations of specificity exist: there are parasites attacking many plant species, only one species, or only a few cultivars of a given species. We shall mainly deal with the last mentioned kind of specificity, viz that between cultivars and races of a single parasitic species.

Cultivar-specific resistance.

Cultivar-specific resistance is observed with many biotrophic and obligate parasites. Flor [10, 11] who studied the inheritance of resistance and virulence in flax, *Linum usitatissimum*, and the flax rust fungus, *Melampsora lini*, respectively, introduced the gene-for-gene hypothesis. This hypothesis is now thought to hold in many other host-parasite interactions [7].

The gene-for-gene concept states that for each gene conditioning avirulence in the parasite there is a corresponding gene conditioning resistance in the host plant. The gene-for-gene concept implies that specificity is associated with the incompatible interaction. Hypersensitivity, callose deposition, lignification and phytoalexin accumulation are host responses which are always strongly associated with incompatibility [8, 16, 17].

Keen and coworkers [12, 13, 14] and Bruegger & Keen [6] claim that in the interactions *Phytophthora megasperma* var. *sojae*-soybean and *Pseudomonas glycinea*-soybean, respectively, the accumulation of the phytoalexin glyceollin is the cause of resistance. Accumulation of glyceollin would be specifically induced by race-specific molecules (elicitors) produced by avirulent races. Ayers *et al.* [3, 4, 5] and Albersheim & Valent [2], however, reported that elicitors

isolated from culture filtrates and cell walls of *Phytophthora megasperma* var. *sojae* were neither race nor cultivar-specific with respect to the accumulation of the phytoalexin glyceollin. Similar results were obtained by Anderson [1] in the interaction *Colletotrichum lindemuthianum*-bean.

The question whether elicitors of phytoalexin accumulation and other possible host defence responses like hypersensitivity, callose deposition or lignification are race-specific or not, should be answered in order to judge whether these responses are the cause or merely the consequence of resistance.

Aim and outline of the present study.

At the time we started this physiological study of cultivar-specific resistance of tomato plants to *Cladosporium fulvum*, the theory about specific elicitors of phytoalexin accumulation had been introduced by Keen [14]. This theory gave a new impulse to research on (i) the possible role of phytoalexins in cultivar-specific resistance, and (ii) the mechanism of induction of phytoalexins.

The host-pathogen interaction *Cladosporium fulvum*-tomato was thought to be an suitable system for study. Moreover, Van Dijkman & Kaars Sijpesteijn [18, 19, 20] had reported at that time most interesting data about a biochemical mechanism for the gene-for-gene resistance of tomato to *Cladosporium fulvum*. They claimed that high molecular weight components present in culture filtrates of avirulent races of *Cladosporium fulvum* increased the rate of leakage of ^{32}P from labelled leaf discs of resistant tomato varieties.

However, we could not confirm their results neither with tomato leaf discs, nor with tomato leaf mesophyll protoplasts. It is true that *Cladosporium fulvum* indeed does produce toxins damaging the host plasma membrane, but these appeared neither race nor cultivar-specific. Similar results were obtained by Lazarovits & Higgins [15] and Dow & Callow [9].

These results prompted us to study this host-pathogen combination along another line. In the present work several aspects of cultivar-specific resistance of tomato plants to *Cladosporium fulvum* have been studied.

Ultrastructural changes in both compatible and incompatible interactions were studied in order to obtain a greater understanding of the time course of the resistance response viz localization of the fungus and the killing of the host cells. Such a study might also be helpful in further research on the biochemical nature of the mechanism of cultivar-specific resistance.

An initial approach of the molecular basis of cultivar-specific resistance was to look for differential *de novo* synthesis of polyadenylic acid (poly-A)-containing messenger RNA (m-RNA) coding for *de novo* protein synthesis which

would be required for phytoalexin-mediated disease resistance. Yoshikawa *et al.* [21, 22] had obtained evidence that in disease resistance of soybeans to *Phytophthora megasperma* var. *sojae de novo* poly (A)-containing m-RNA synthesis was associated with *de novo* synthesis of enzymes involved in glyceollin production, a phytoalexin thought to be the cause of resistance in this disease.

It has been investigated whether differential changes in soluble leaf proteins occur in compatible and incompatible interactions. The possible role of phytoalexins with respect to fungal growth inhibition in incompatible interactions was studied. As differential accumulation of phytoalexins occurred in compatible and incompatible interactions it was investigated whether the phytoalexins involved could be induced specifically by elicitors of avirulent races of *Cladosporium fulvum*. The chemical composition and structure of these elicitors were also studied.

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Netherlands Journal of Plant Pathology (1977) 83, 109-122

A LIGHT AND SCANNING-ELECTRON MICROSCOPIC STUDY OF INFECTION OF TOMATO PLANTS BY VIRULENT AND AVIRULENT RACES OF *CLADOSPORIUM FULVUM*

by

P.J.G.M. de Wit

ABSTRACT

Infection of tomato plants by *Cladosporium fulvum* Cooke was studied by using light and scanning-electron microscopy. Races 1.2.3 and 4 of *Cladosporium fulvum* were used, whereas tomato cultivars, carrying the Cf2 gene (susceptible to race 1.2.3 and immune to race 4) and the Cf4 gene (immune to race 1.2.3 and susceptible to race 4) served as differentials. No differences were observed in growth between compatible and incompatible combinations during germination, subsequent formation of runner hyphae and stomatal penetration. Runner hyphae did not show directional growth towards stomata.

Penetration usually occurred on the third or fourth day after inoculation. In compatible combinations the fungus grew intercellularly, often in close contact with spongy mesophyll cells. Under optimal conditions it dit not cause visible damage to plant cells during early stages of infection. Under suboptimal conditions in winter, the host cells often reacted with callose deposition, but growth of the fungus did not appear to be inhibited. Ten to twelve days after inoculation conidiophores emerged through the stomata and produced conidia.

In incompatible combinations fungal growth was arrested one to two days after penetration and confined to stomata and surrounding cells. Very soon the host cells, in contact with the fungus, deposited extensive amounts of callose. Later these cells turned brown and collapsed. At the surface of the host cells, contacted by fungal hyphae, abundant extracellular material could be observed by scanning-electron microscopy.

Removing the epidermis of leaves before inoculation delayed the resistant

response. On stripped leaves the rate of fungal growth was equal for both interactions up to ten days after inoculation, but the incompatible combination lacked sporulation.

INTRODUCTION

Cladosporium fulvum Cooke causes leaf mould of tomato (*Lycopersicon esculentum* Mill.). Many physiological races of the fungus are known (Hubbeling, 1966). In the cultivated tomato there are several genes for resistance, originating from wild tomato species (Kooistra, 1964; Hubbeling, 1968, 1971). A gene-for-gene relationship has been proposed (Day, 1956; Kooistra, 1964). The genes for resistance condition different reactions. Plants carrying resistance genes Cf2 and Cf4 react after infection by an avirulent race with very restricted lesions, not visible with the naked eye. This type of high resistance is called immunity (Hubbeling, 1971; Lazarovits & Higgins, 1976a, 1976b). In contrast, plants carrying resistance genes Cf1 and Cf3 permit some fungal growth and even some sporulation. Bond (1938) studied the combination tomato-*Cladosporium fulvum* by light microscopy. Since then, many new races have appeared and many genes for resistance were introduced by breeding.

In view of this, it would be of interest to study ultrastructural characteristics of different host-pathogen combinations. While this study was underway new histological and ultrastructural data on the interactions of *Cladosporium fulvum* race 1 with susceptible (Cf0 gene), resistant (Cf3 gene for resistance) and immune (Cf2, Cf4 genes for resistance) tomato plants were published (Lazarovits & Higgins, 1976a, 1976b). The present study is meant as an introduction to a physiological study on resistance and susceptibility of tomato plants to various physiological races of the fungus.

In this study, races 1.2.3 and 4 were used, whereas "Vetomold" (carrying the Cf2 gene for resistance, susceptible to races 1.2.3 and immune to race 4) and "Purdue 135" (carrying the Cf4 gene for resistance, immune to race 1.2.3 and susceptible to race 4) served as differentials. Later on, also near-isogenic lines of "Moneymaker", carrying resistance genes Cf2 and Cf4, were used. Preliminary results of this study have been published elsewhere (De Wit and Hijwegen, 1976).

MATERIALS AND METHODS

Plants.

Seed of tomato "Purdue 135", "Vetomold" and the near-isogenic lines of "Moneymaker" carrying the resistance genes Cf2 and Cf4 (generously supplied by Mrs I. Boukema, IVT, Wageningen) were sown in trays with peat soil (Trio no. 17); seedlings were transplanted in pots (diameter 18 cm) after two weeks. Plants were kept in a greenhouse between 20 °C and 25 °C at 60% relative humidity. Light intensity was kept between 10000 and 15000 lux for 12 hours each day by shadowing the greenhouse in the summer with lime if necessary and supplementing with incandescent light from HPLR-400 W lamps (Philips) during the winter.

The fungus.

Monospore cultures of *Cladosporium fulvum* race 1.2.3 and race 4 (kindly supplied by Mr N. Hubbeling, IPO, Wageningen) were subcultured on potato-dextrose agar at 22 °C. Twice a year races were reisolated from susceptible tomato varieties, to ensure that they retained their virulence. Conidia from three-week-old cultures on potato-dextrose agar were used for inoculation.

Inoculation.

a) Intact plants. Tomato plants of six to seven weeks old were used for inoculation. The fourth or fifth leaf was inoculated at the lower side by spraying with a conidial suspension in water (10^6 conidia ml^{-1}). After drying plants were incubated in plastic boxes. During the first day relative humidity was kept at 100% by keeping the boxes completely closed; afterwards, relative humidity was maintained between 85% and 100%, by slightly opening the boxes at day time and closing them at night. Temperature varied between 19 °C and 24 °C and light intensity between 10000 and 15000 lux.

b) Excised stripped leaves. In a few experiments excised leaves, with the cut ends of the petioles covered with moistened cotton wool, were placed in Petri dishes (diameter 15 cm) containing moistened filter paper and incubated in a climate room at 20-24 °C. Light intensity was 10000 lux for 16 hours a day, supplied by white fluorescent tubes (Philips 60 W). The lower epidermis of the leaves was removed prior to inoculation, so that conidia and germinating fungal hyphae were in direct contact with the mesophyll cells of the leaves.

Light, fluorescence and scanning-electron microscopy.

Samples were taken at different times after inoculation. To assess germination and penetration of the fungus, epidermal strips of inoculated leaves were examined under the light microscope after staining in a solution of cotton blue

in lactophenol.

To study growth of the fungus inside the leaf, leaf discs were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7, and dehydrated in an ethanol series. For ordinary light microscopy fixed leaves were stained in lactophenol-cotton blue and for fluorescence in 0.1% aniline blue in 1/15 M K_3PO_4, pH 12.2, for at least three hours. Observations were made under a Wild microscope after the method of Shimomura & Dijkstra (1975). Starch was stained with a solution of iodine and potassium iodide.

For scanning-electron microscopy, leaf discs were fixed in the same way as described for light microscopy (a few samples were postfixed in 2% OsO_4). After dehydration in ethanol leaf discs were gradually transferred to amyl acetate in a series of ethanol and amyl acetate mixtures. Discs were critical point dried (POLARON critical point apparatus) and coated with a thin layer of gold (Balzers coating unit with attachment for gold sputtering, type BAE 301). They were then examined in a scanning-electron microscope (type Jeol JSM U_3 or Hitachi). In order to observe penetration and subsequent colonization of the leaf by the fungus the lower epidermis of inoculated leaves was removed before fixation. Penetration through the stomata was observed at the lower side of the stripped epidermis and colonization of the leaf in the stripped leaves themselves.

RESULTS

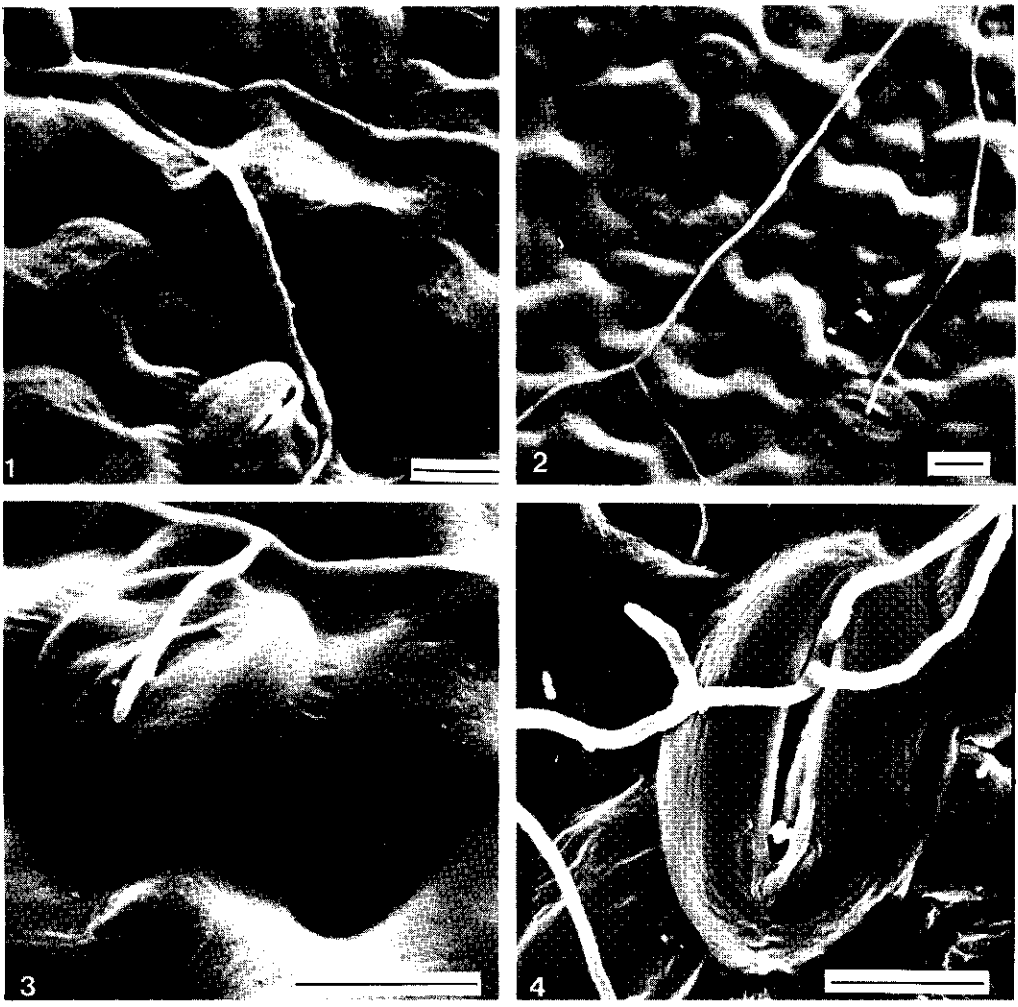
Conidial germination and stomatal penetration.

In Table 1 percentages of conidial germination and subsequent stomatal penetration

Table 1. Percentage of conidial germination (g) and stomatal penetration (p) by race 1.2.3 on "Vetomold" (susceptible) and "Purdue 135" (immune). Inoculations were carried out during winter (November-February) and summer (June-August).

Days after inoculation	Inoculated during winter				Inoculated during summer			
	"Vetomold"		"Purdue 135"		"Vetomold"		"Purdue 135"	
	g	p	g	p	g	p	g	p
2	55	2	47	1	43	7	47	4
3	77	2	52	3	51	10	58	7
4	91	6	79	5	76	33	67	35
5	91	11	82	13	83	39	88	46
6	85	19	88	15	90	74	87	61

In all figures, bar represent 10 µm.
Fig. 1. "Vetomold" + race 1.2.3, five days after inoculation; runner hyphae passing by stomata.
Fig. 2. "Purdue 135" + race 1.2.3, seven days after inoculation; hypha entering a stoma.
Fig. 3. "Vetomold" + race 1.2.3, seven days after inoculation; hypha entering a stoma by a side branch.
Fig. 4 "Purdue 135" + race 4, two days after inoculation; hypha passing over a stoma.



by race 1.2.3 on both varieties are compared. With respect to germination and penetration there were no significant differences between compatible and incompatible interactions. The percentage of penetration of germinated conidia was higher during summer than during winter. During winter plants were supplied with artificial light. For race 4 results were similar. Although penetration

occurred through stomata only, there was no directional growth of runner hyphae towards stomata (Fig. 1). Scanning-electron microscopy revealed that germinated hyphae grew over and along stomata already one day after inoculation but penetration was usually observed not earlier than two to three days after inoculation (Fig. 2 and 3). Very often hyphae were growing over stomata without entering them (Fig. 4). Possibly, during the first days of incubation, when relative humidity was near 100%, conidial germination was favoured, but penetration prevented, because most stomata were closed under these circumstances. Variable relative humidity did stimulate stomatal opening, which was necessary for fungal penetration. The hyphae penetrated mostly without formation of an appressorium, directly or by means of a side branch (Fig. 2 and 3). In incompatible interactions it was often seen that the fungus grew out of the stoma again, which it had entered (Fig. 5). In successful penetrations of incompatible combinations often extracellular material was deposited on the surface of the stoma (Fig. 5). This extracellular material may extend into the stomatal cavity as could be observed at the inner side of the stripped epidermis (Fig. 6).

Fungal growth and plant response inside the leaf in compatible and incompatible interactions.

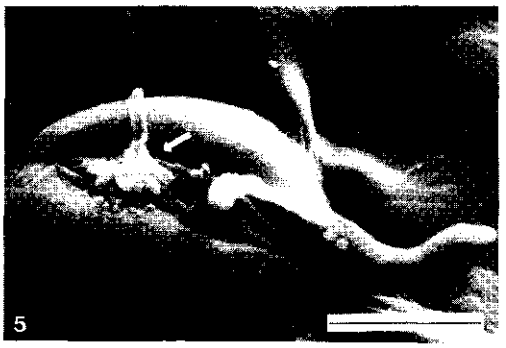
On the leaf surface no differences in growth between virulent and avirulent races were observed, but as soon as the fungus had entered the stoma, histological differences between both combinations could be detected.

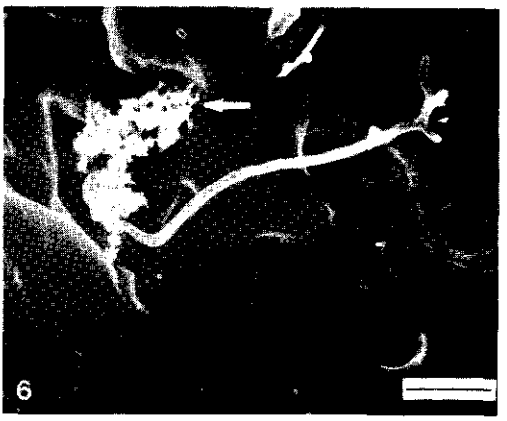
Light microscopy.

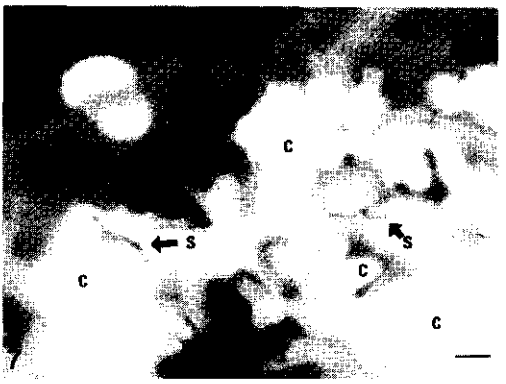
Epidermal and spongy mesophyll cells around stomata, which were penetrated stained more intensely with cotton blue in the incompatible combination. Very often, the penetrated stoma itself did not contain starch and was coloured brown in the incompatible interactions as a result of a hypersensitive response. This reaction was rarely seen in compatible interactions.

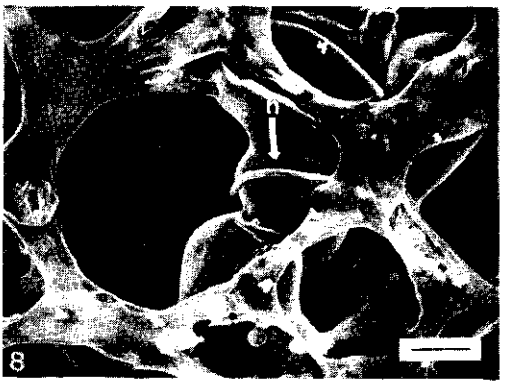
In fixed leaves, fungal growth inside the leaf could be examined rather easily. Four to five days after inoculation, that means one to two days after penetration, fungal growth in incompatible interactions was arrested while in compatible interactions it was abundant. After penetration the fungal hyphae were distinctly thickened in compatible as well as in incompatible interactions. Fungal growth inside the leaf was exclusively intercellular. After fungal contact the spongy mesophyll cells reacted differently in the two types of interactions. In incompatible interactions spongy mesophyll cells turned brown after

Fig. 5. "Purdue 135" + race 1.2.3, seven days after inoculation; hypha entering and leaving a stoma again; extracellular material present at the stoma (arrow).
Fig. 6. "Purdue 135" + race 1.2.3, fourteen days after inoculation; inner side of stripped epidermis; short penetrated hypha; note extracellular material (arrow).
Fig. 7. "Purdue 135" + race 1.2.3, callose deposition (C) around penetrated stomata (S) observed under fluorescence microscope.
Fig. 8. "Purdue 135" + race 4, seven days after inoculation; hypha (H) in close contact with spongy mesophyll cells.









hyphal contact, a phenomenon rarely seen in compatible interactions.

Fluorescence microscopy.

a) Intact leaves. Fluorescence microscopy was used to investigate whether compatible and incompatible combinations reacted differently with regard to callose deposition. Callose deposition is often regarded as a reaction of plants to wounding (Currier, 1957; Nims *et al.*, 1967). In fungal, bacterial and virus

diseases, callose formation has been suggested to be correlated with incompatibility (Heath, 1971, 1972, 1974; Shimomura & Dijkstra, 1975). Usually it is deposited as a thick layer between cell membrane and cell wall and is often looked upon as a response occurring before hypersensitive cell death.

In Table 2 callose deposition in leaves of "Vetomold" and "Purdue 135" after inoculation with race 1.2.3 and 4 are compared. The experiment was carried out during winter. From this table it can be concluded that callose deposition was not specific for the incompatible interaction. In both combinations callose was deposited profusely. In incompatible interactions fungal growth was limited to the fluorescent area. In compatible interactions the fungus outgrew the fluorescent areas; the growing mycelium did not give rise to further callose formation. Therefore, callose formation seemed to be an early reaction of the host after initial fungal contact, irrespective of the type of interaction involved. This experiment was repeated during summer with the same cultivars and with two near-isogenic lines of "Moneymaker" carrying Cf2 and Cf4 genes for resistance. Results of the latter experiment are presented in Table 3. Here, callose formation was almost specific for the incompatible interaction. In Fig. 7 a typical appearance of callose deposition in an incompatible interaction, as seen under the fluorescence microscope, is shown.

b) Stripped leaves. Stripped leaves, inoculated with conidia of both races fluoresced already 12 hours after inoculation. Fluorescence had increased at 24 hours and 48 hours after inoculation. The non-inoculated stripped leaves did not show appreciable fluorescence.

Although results in Table 3 suggested that callose deposition might be causally related to resistance, the experiments with stripped leaves showed that callose deposition seemed to be a consequence rather than a cause of resistance.

On stripped leaves of immune reacting combinations fungal growth was not inhibited. Hence, after removal of the epidermis a resistant leaf became more or less susceptible.

Scanning-electron microscopy.

a) Intact leaves. SEM studies provided some more details about fungal growth especially inside the leaf. Inside the leaf the fungus could be observed by removing the lower epidermis at different times after inoculation. The fungus grew exclusively intercellularly without formation of haustoria. In compatible interactions fungal growth was abundant, in many cases in close contact with

Table 2. Callose deposition in incompatible and compatible combinations. Experiments were carried out during winter (November-February).

	Host-parasite combinations											
	4 days after inoculation				6 days after inoculation				8 days after inoculation			
	A*	B	C	D	A	B	C	D	A	B	C	D
Number of penetrations	11	6	5	-	13	15	11	16	13	20	6	6
Length of penetrated hypha, expressed in cell diameter	1/4-2	1/2-2	1/2-2	-	1/4-3	1/2-5	1/2-10	1/2-3	1/4-3	1/2-∞**	1/2-∞	1/2-3
Number of penetrations with callose deposition	10	4	3	-	5	5	5	11	21	0	2	3
Percentage of penetrations with callose deposition	91	67	60	-	38	33	45	62	70	0	33	50

*A = "Purdue 135", inoculated with race 1.2.3.
B = "Vetomold", inoculated with race 1.2.3.
C = "Purdue 135", inoculated with race 4.
D = "Vetomold", inoculated with race 4.
- = no observations.
**∞ = beyond limit of microscopic field (= 300 µm diam).

Table 3. Callose deposition in incompatible and compatible combinations. Experiments were carried out during summer (June-August)

	Host-parasite combinations											
	4 days after inoculation				6 days after inoculation				8 days after inoculation			
	A*	B	C	D	A	B	C	D	A	B	C	D
Number of penetrations	30	30	30	30	30	30	30	30	30	30	30	30
Length of penetrated hypha, expressed in cell diameter	1/4-3	1/4-3	1/4-3	1/4-3	1/4-3	1/4-3	1/2-10	1/2-10	1/4-3	1/4-3	1/2-∞**	1/2-∞
Number of penetrations with callose deposition	2	1	0	0	18	19	1	0	22	25	1	3
Percentage of penetrations with callose deposition	6	3	0	0	60	63	3	5	73	83	3	10

*A = "Purdue 135", inoculated with race 1.2.3.
B = "Near-isogenic line Cf4", inoculated with race 1.2.3.
C = "Vetomold", inoculated with race 1.2.3.
D = "Near-isogenic line Cf2", inoculated with race 1.2.3.
**∞ = Beyond limit of microscopic field (= 300 µm diam.).

spongy mesophyll cells (Fig. 8 and 9), but in others not at all. As far as could be observed in SEM, host cells did not react after fungal contact. Nine to ten days after inoculation formation of stroma could be observed beneath the stomata. Later on, conidiophores emerged through stomata (Fig. 10) and produced conidia.

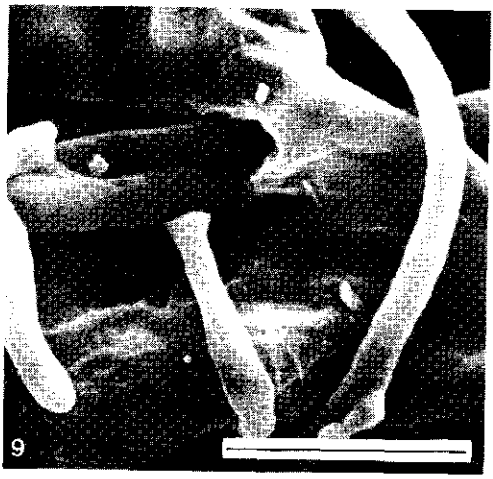
In incompatible interactions mycelium was rarely observed between the spongy mesophyll cells. As could be seen at the inner side of stripped epidermis, the length of the penetrated hyphae varied to a certain extent, but never exceeded a length of four to five times the diameter of an epidermal cell (Fig. 6 and 11). The hyphae in incompatible interactions were often swollen and curled. Sometimes amorphous material was deposited around the penetrated hyphae (Fig. 11). Hyphae in contact with mesophyll cells appeared to be collapsed; the mesophyll cells were often also collapsed or showed abnormal extracellular material at the surface of the cell wall (Fig. 12). This extracellular material occurred as "bubbles" and was probably produced by the host cells after fungal contact. It was most frequently found on the spongy mesophyll cells, but also at the inner side of epidermis cells, which were contacted by a hypha.

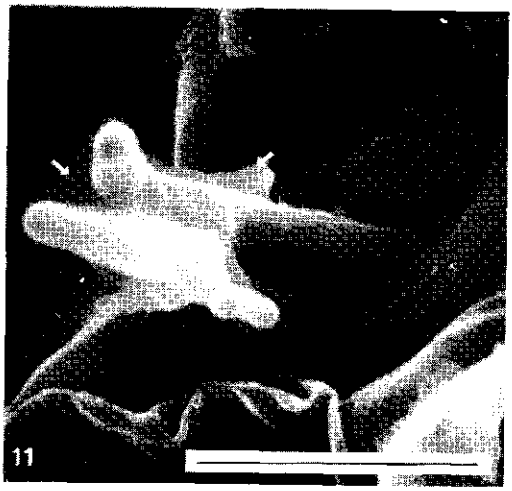
b) Stripped leaves. As could be concluded from these experiments, compatibility or incompatibility became apparent after the fungus had entered the tomato leaves. In the next experiments the epidermis was removed very gently before leaves were inoculated with conidia. There was no difference in mycelial growth on stripped leaves of immune and susceptible reacting plants. The mesophyll cells, which were contacted by hyphae of germinated conidia did not appear to be collapsed in both combinations. However, the so-called "bubbles" occurred very frequently, mainly in the incompatible interactions (Fig. 13).

On stripped leaves two types of hyphae could clearly be distinguished: 1) Thin runner hyphae, which are normally only produced at the leaf surface; 2) Hyphae with two to three times the diameter of runner hyphae, normally only produced inside the leaf immediately after penetration of a stoma (Fig. 14).

Although under natural circumstances thick hyphae were formed immediately after runner hyphae had penetrated a stoma, on stripped leaves where hyphae of germinated conidia were in direct contact with mesophyll cells, runner hyphae were formed first. The thickened hyphae were observed only a few days after inoculation of the stripped leaves. They were in close contact with the spongy mesophyll cells. Thus, it may be concluded that contact with mesophyll cells was not the only stimulus that triggered formation of thick hyphae.

Fig. 9. "Purdue 135" + race 4, ten days after inoculation; hypha in close contact with spongy mesophyll cells.
Fig. 10. "Purdue 135" + race 4, twelve days after inoculation; young conidiophores emerging from a stoma.
Fig. 11. "Purdue 135" + race 1.2.3, fourteen days after inoculation; inner side of stripped epidermis; penetrated hypha (H) in stoma (S) short and swollen; note amorphous material around hyphal tips (arrow).
Fig. 12. "Purdue 135" + race 1.2.3, twelve days after inoculation; collapsed mycelium and extracellular material present at the cell wall of mesophyll cells (arrow).





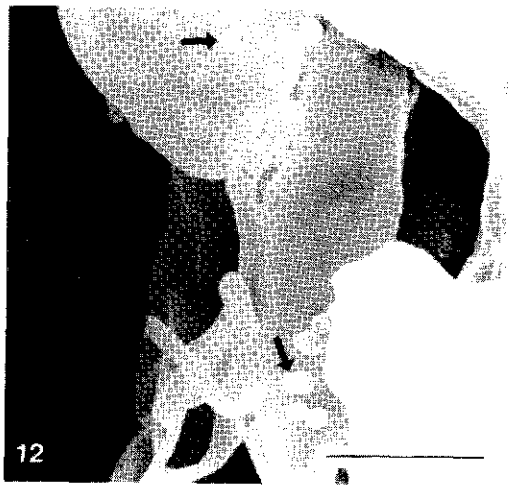
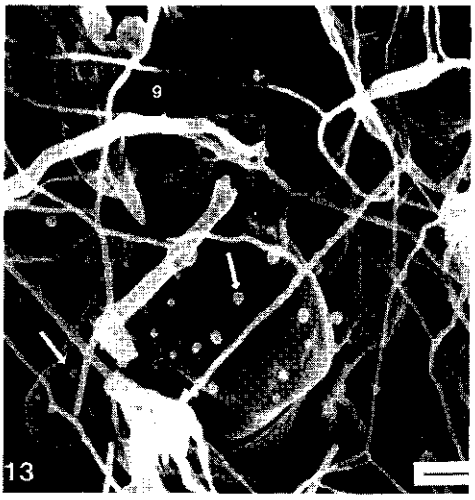
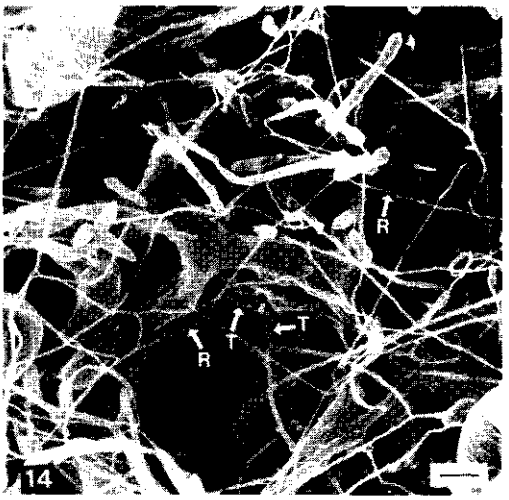


Fig. 13. "Purdue 135" + race 1.2.3, nine days after inoculation; extracellular material present (arrow); abundant growth of mycelium on stripped leaf.
Fig. 14. "Purdue 135" + race 1.2.3, nine days after inoculation; runner hyphae (R) and thickened hyphae (T) on stripped leaf.





DISCUSSION

On the leaf surface *Cladosporium fulvum* behaved very similarly to *Cercospora beticola* (Rathaiah, 1976). There was no directional growth of the runner hyphae towards the stomata and penetration occurred three to four days after inoculation. Rathaiah found, that for successful penetration stomata need not te be open. Zoospores of fungi like *Pseudoperonospora humuli* and *Plasmopara viticola* show a positive chemotaxis to stomata (Royle & Thomas, 1973) and rust hyphae show directional growth of germ tubes towards stomata (Heath, 1974). Light, fluorescence and scanning-electron microscopy revealed no differences between virulent and avirulent races of *C. fulvum* with respect to conidial germination and stomatal penetration. This is in agreement with observations described for other host-pathogen combinations (Skipp & Deverall, 1972; Skipp & Samborski, 1974).

Cell browning and cell collapse are suggested to be a consequence rather than a cause of the initial resistant reaction. Similar conclusions were drawn

by other workers for other host-pathogen interactions (Ogle & Brown, 1971; Király *et al.*, 1972; Mayama *et al.*, 1975).

In plants inoculated during summer, callose deposition was observed almost exclusively in incompatible interactions, as described also by Lazarovits & Higgins (1976a, 1976b). Inoculations carried out during winter showed callose deposition in nearly equal amounts in both interactions. Also when plants were inoculated with conidia of two to three-month-old cultures, callose deposition was the same for both interactions. Hubbeling (personal communication) observed, that susceptible plants produced many necrotic spots when inoculated with conidia of old cultures. Thus, callose deposition is influenced by external conditions. As soon as a runner hypha had penetrated a leaf its diameter increased two to three-fold. It is not known how the fungus is feeding itself inside the leaf. According to Van Dijkman (1972) the fungus does not produce polygalacturonases or cellulases in significant amounts in vitro. This oberva-tion was confirmed by us, except for C_x-cellulase, which was produced by the fungus when grown on several media, including cell walls of tomato mesophyll cells. The fungus is probably living on substances leaching from the mesophyll cells into the intercellular space. It is known that sugars and other photosynthetic products synthesized in the mesophyll cells leach into the free space and are transported to the small veins by so-called transfer cells (Kursanov & Brovchenko, 1970). The extracellular material at the surface of mesophyll cells, found in incompatible interactions was also reported by Lazarovits & Higgins (1976a, 1976b). They observed this extracellular material more frequently in resistant plants carrying the Cf3 gene for resistance than in plants carrying the Cf2 and Cf4 genes for resistance. In our case this extracellular material was found in plants carrying the Cf2 and Cf4 genes for resistance. According to Lazarovits & Higgins (1976a) the main components of this material were polyphenols.

There was a significant difference in mycelial growth in incompatible interactions between intact leaves and leaves from which the lower epidermis had been stripped off prior to inoculation. For stripped leaves expression of immunity appeared to be delayed in time. Fungal growth was rather abundant and the only difference between compatible and incompatible interactions was lack of sporulation in the incompatible combinations. It seems possible that the early resistant response is triggered in the penetrated stoma, after recognition of the fungus by the guard cells, because very often these guard cells were the first to react after fungal contact.

The question how fungal growth is inhibited in the incompatible interaction remains still to be solved. These problems will be the subject of further studies.

ACKNOWLEDGEMENTS

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DIFFERENTIAL CHANGES IN SOLUBLE TOMATO LEAF PROTEINS AFTER INOCULATION WITH VIRULENT AND AVIRULENT RACES OF *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)

by

P.J.G.M. de Wit and J. Bakker

ABSTRACT

Changes in the soluble protein consitution of leaves of near-isogenic lines of tomato, carrying resistance gene Cf4 or Cf5 to *Cladosporium fulvum* (syn. *Fulvia fulva*), were investigated by polyacrylamide gel electrophoresis after inoculation with race 4 or 5 of this fungus. A protein having R_F 29 on 7% polyacrylamide gels appeared more rapidly in the two incompatible combinations (Cf4 + race 5; Cf5 + race 4) than in the compatible ones (Cf4 + race 4; Cf5 + race 5). The protein which is presumably of host origin was synthesized *de novo* after inoculation with *Cladosporium fulvum*, but appeared also in non-inoculated leaflets of a compound leaf opposite to the inoculated ones. However, in control leaves of healthy non-inoculated plants the protein was absent or present in very low concentrations.

Although the appearance of the protein was strongly associated with the hypersensitive response in incompatible interactions, at the tested concentrations, it did not inhibit hyphal growth of *Cladosporium fulvum* in tomato leaf. The protein did show a faint positive reaction with Schiff's stain for carbohydrates.

Besides the mentioned protein, a significant increase of peroxidase isoenzymes occurred in the incompatible interactions compared with the compatible ones.

INTRODUCTION

Tomato plants carrying Cf2, Cf4 or Cf5 genes for resistance to *Cladosporium fulvum* (syn. *Fulvia fulva*) reacted with a hypersensitive response after

inoculation with avirulent races of this pathogen [4, 11, 12]. In incompatible combinations hyphal growth in tomato leaves was inhibited three to five days after inoculation, shortly after stomatal penetration. At this time, accumulation of phytoalexins was observed [5]. Penetrated hyphae were often enclosed by extracellular material, while the host plant reacted with extensive deposition of callose [4, 11]. After prolonged incubation some of these phenomena also occurred in compatible interactions [4, 5]. These results indicate that resistance in the combination *Cladosporium fulvum*-tomato is an active process.

Yoshikawa *et al.* [25, 26] reported that *de novo* synthesis of polyadenylic acid (Poly-A) containing messenger RNA (m-RNA) and protein was required for phytoalexin-mediated disease resistance in soybeans against *Phytophthora megasperma* var. *sojae*. Yamamoto *et al.* [24] showed that *de novo* protein synthesis was linked with resistance in oats against *Puccinia coronata*. They also showed that treatment of oat leaves with blasticidin S (BcS), a protein synthesis inhibitor, turned an incompatible interaction into a compatible one.

In virus-host interactions the appearance of host-specific proteins has been reported in hypersensitively reacting plants [18, 22].

The present study was undertaken to investigae whether new host-specific or fungal-specific proteins could be detected at the establishment of the incompatible interaction of *Cladosporium fulvum* and tomato. This paper reports the results of a study of soluble leaf proteins by electrophoretic techniques.

MATERIALS AND METHODS

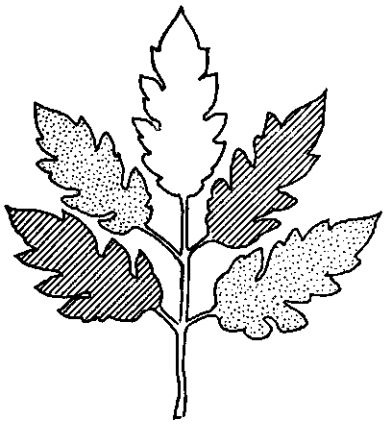
Plants.

Near-isogenic lines of Moneymaker carrying the Cf4 gene for resistance (susceptible to race 4 and immune to race 5) and the Cf5 gene for resistance (immune to race 4 and susceptible to race 5) served as differentials. They were grown as described before [5].

Fungi.

Monospore cultures of races 4 and 5 of *Cladosporium fulvum* were subcultured on potato dextrose agar (PDA) as described before [5].

Fig. 1. Inoculation procedure for leaflets of a compound tomato leaf. Stipple inoculated; shaded, control.



Inoculation.

The fourth fully expanded compound leaf of six to seven-week-old tomato plants was used for inoculation. Two opposite leaflets of a compound leaf were shaded with aluminium foil and two other opposite leaflets were inoculated on the abaxial side by spraying with a conidial suspension of *Cladosporium fulvum* in water containing 10^7 conidia ml^{-1} (Fig. 1). After inoculation, the plants were allowed to dry and sprayed twice again to achieve a dense and homogeneous inoculum. Inoculated plants were incubated as described before [5].

Microscopic observation.

Samples of inoculated leaflets used for protein extraction and subsequent gel electrophoresis were examined by light microscopy to assess leaf colonization by *Cladosporium fulvum* in compatible and incompatible interactions. Microscopic observations were carried out as described before [4, 5]

Extraction and gel electrophoresis of soluble leaf proteins.

Leaf samples, taken at several times after inoculation, were homogenized at 5 °C in 0.1 M Tris/HCl buffer, pH 8, containing 0.5 M saccharose, 0.1% ascorbic acid and 0.1% L-cysteine (2.5 ml buffer/g leaf tissue) as described by Staples & Stahmann [17]. The homogenate was centrifuged at 40000 g for 1 hour. The supernatant containing the soluble leaf proteins was subjected to several

electrophoretic procedures. Discontinuous polyacrylamide gel electrophoresis was performed according to Davis [3] using a 2.5% stacking gel and a 7% separating gel. Electrophoresis was carried out at 5 °C in buffer, pH 8.3, containing 0.005 M Tris and 0.04 M glycine at 2.5 mA/tube for about 30 min, followed by 4 mA/tube for another 90 min. Bromophenol blue was used as a marker. Gels were stained in a saturated solution of amido black in 5% trichloroacetic acid (TCA) for one night and destained in 7% acetic acid. Gels were scanned at 620 nm in a Beckman spectrophotometer (model 25) equipped with a gel scanner accesory. The positions of the protein bands were expressed by a R_F value; the marker, bromophenol blue was given R_F 100.

Preparative discontinuous polyacrylamide slab gel electrophoresis according to the method of Davis [3] was performed in 7% slab gels. From these preparative slab gels, two longitudinal strips were removed and stained in amido black to reveal the protein pattern. The protein band to be investigated was then excised from the main gel and boiled for two min in a small volume of 0.025 M Tris/HCl buffer, pH 6.8 containing 2% sodium dodecyl sulphate (SDS), 10% glycerol and 2% 2-mercapto ethanol. The sample was then subjected to polyacrylamide slab gel electrophoresis according to the method of Laemmli [13] using a 5% stacking gel and a 10% separating gel. Electrophoresis was carried out at 5 °C in buffer, pH 8.3, containing 0.025 M Tris, 0.192 M glycine, and 0.1% SDS at 20 mA for about six hours. Gels were stained with Coomassie brilliant blue (15 mg Coomassie brilliant blue, dissolved in a solution of methanol, water and acetic acid (45 : 45 : 9, v/v/v) for two hours and destained in a solution of methanol, water and acetic acid (10 : 35 : 175, v/v/v). Apparent molecular weights were estimated from the positions on the SDS slab gels of reference proteins including lactate dehydrogenase (LDH), bovine serum albumin (BSA), malate dehydrogenase (MDH) and isocitrate dehydrogenase (ICDH).

Labelling of soluble leaf proteins with $^{35}SO_4^{2-}$ and ^{3}H-amino acids.

Inoculated compound leaves (with inoculated and control leaflets) were cut and placed in 1 ml of Hoagland solution, containing 25 µCi of [^{3}H(G)] L-amino acids or 25 µCi of $^{35}SO_4^{2-}$ (The Radiochemical Centre, Amersham). After uptake of the radiochemicals the leaves were placed for another 20 hours in Hoagland solution. Then the leaves were homogenized and samples were prepared for electrophoresis as described before. Radioactivity was determined in the total supernatant fraction as well as in the TCA-insoluble fraction; the latter was obtained by adding an equal volume of 30% TCA to the supernatant fraction, collecting the

precipitate by centrifuging for 10 min at 20000 g, washing the pellet with 15% TCA and dissolving it in 0.5 N NaOH before counting. One hundred µl samples of the supernatant fractions were used for electrophoresis. After electrophoresis the gels were stained, scanned and sliced transversely in 1 mm discs by a gel slicer (Mickle). Each disc was dissolved in 1 ml 30% H_2O_2 in a counting vial and incubated overnight at 60 °C. To all samples 10 ml of scintillation mixture (Lumac) was added before the radioactivity was measured in a liquid scintillation spectrometer (Mark III, Nuclear Chicago).

Detection of glycoproteins and enzymes on disc gels.

Glycoproteins were detected with Schiff's base reagent as described by Zacharius *et al.* [27]. Peroxidase and catalase were detected by the method of Macko *et al.* [15]; polyphenol oxidase and ribonuclease by the methods described by Van Loon [19]; acid phosphatase, acetyl esterase, malate dehydrogenase, glucose-6-phosphate dehydrogenase and glutamate dehydrogenase by the method of Sako & Stahmann [16].

Protein determination.

Protein was measured according to the method of Lowry *et al.* [14] as modified by Hartree [9] with BSA as a standard.

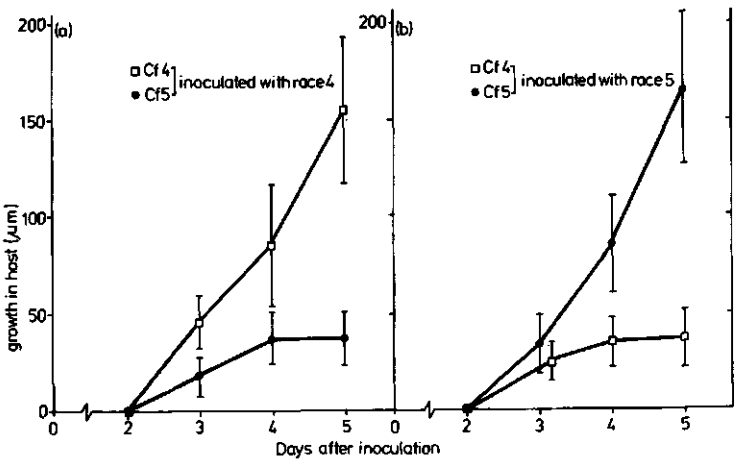
RESULTS

Changes in soluble leaf protein constitution of near-isogenic lines of tomato after inoculation with virulent and avirulent races of Cladosporium fulvum.

Whole leaves. Our main objective was to study changes in soluble leaf proteins at early stages of infection, before or at the time of the establishment of incompatible interactions. Fungal growth in resistant and susceptible near-isogenic lines of tomato was estimated at various times after inoculation (Fig. 2). Until penetration runner hyphae of virulent and avirulent races grew at a similar rate on leaves of susceptible and resistant lines, respectively. Soon after stomatal penetration, three to four days after inoculation, growth of avirulent races was arrested. This indicates that resistance in terms of fungal growth inhibition was expressed between three and four days after inoculation.

Plate 1 shows the electrophoretic patterns of soluble leaf proteins of near-isogenic lines Cf4 and Cf5, at 3, 4, 5 and 7 days after inoculation with races 4 or 5 of *Cladosporium fulvum*, respectively. The major difference between

Fig. 2. Growth of race 4 (a) and race 5 (b) of *Cladosporium fulvum* in tomato leaves of near-isogenic lines Cf4 (□) and Cf5 (●). Values are the mean (± standard deviation) of 25 measurements.



compatible (Plate 1B, Cf4/race 4; Place 1C, Cf5/race 5) and incompatible (Plate 1A, Cf5/race 4; Plate 1D, Cf4/race 5) interactions was the greater staining density of a protein band having R_F 29 in both incompatible interactins at 3, 4 and 5 days after inoculation. At seven days after inoculation the staining density of band R_F 29 was similar in both types of interactions.

In control leaflets of healthy non-inoculated plants only a faint band R_F 29 was present (Plate 1Ac, Bc, Cc, Dc). Also in control leaflets opposite to inoculated ones only a faint band R_F 29 was present during the first seven days after inoculation (results not shown). However, seven to ten days after inoculation in these control leaflets (irrespective of whether they were inoculated with a virulent or avirulent race) band R_F 29 was also present (Plate 1Ac10, Bc10, Cc10, Dc10), though its staining density was less intense than in inoculated leaflets.

Besides protein band R_F 29, at seven days after inoculation protein band R_F 35 also increased considerably in intensity, especially in both compatible interactions. Coelectrophoresis of soluble mycelial proteins revealed that band R_F 35 is very likely of fungal origin.

Lower epidermis. Fungal growth in incompatible interactions is strongly inhibited after stomatal penetration, and the stomata and surrounding cells are often the first to react by deposition of callose [4, 11] and production of

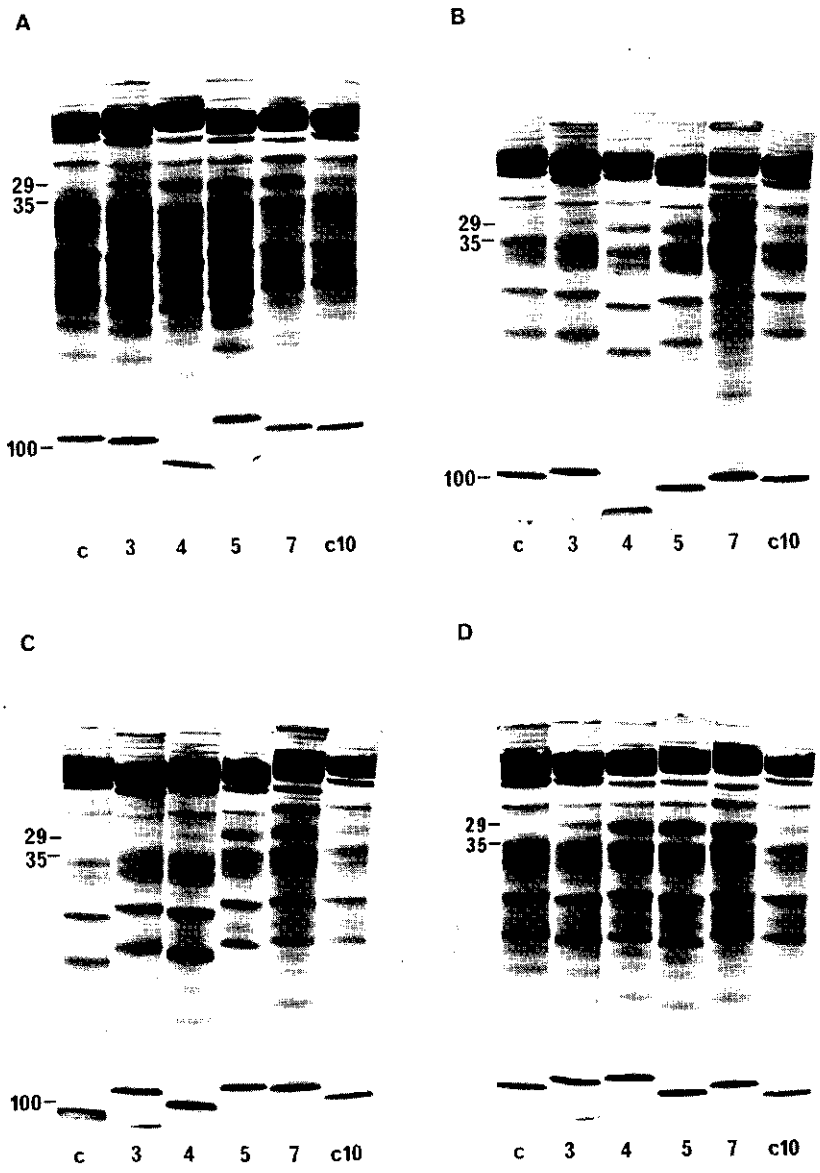


Plate 1. Protein band patterns of soluble leaf proteins of near-isogenic lines of Cf4 and Cf5, at various times after inoculation with race 4 or 5 of *Cladosporium fulvum*. Electrophoresis was carried out on 7% polyacrylamide gels. Each set of gels represents from left to right: c control of healthy plants; samples taken 3, 4, 5 and 7 days after inoculation; c10 control leaflets opposite to inoculated leaflets sampled ten days after inoculation; A, Cf5/race 4; B, Cf4/race 4; C, Cf5/race 5; D, Cf4/race 5. R_F 29, R_F 35 and R_F 100 are indicated. The development of the infection at the various sampling dates is shown in Fig. 2.

phytoalexins [5]. For this reason, soluble proteins of lower epidermis strips from leaves which had been inoculated with virulent and avirulent races of *Cladosporium fulvum* were subjected to electrophoresis. Results were comparable with those shown for whole leaves. Also here protein band R_F 29 appeared first and was most intense in both incompatible interactions (Cf5/race 4; Cf4/race 5).

Incorporation of $^{35}SO_4^{2-}$ and ^{3}H-amino acids in soluble protein fractions.

In order to examine whether the resistance response was mediated by *de novo* protein synthesis, especially of protein band R_F 29, leaves of near-isogenic lines were placed in a solution containing $^{35}SO_4^{2-}$ or a mixture of [^{3}H(G)]L-amino acids at three days after inoculation. One day after feeding with the label, leaves were homogenized and the radioactivity in the total soluble leaf protein fraction and in the TCA-insoluble leaf protein fraction was determined. Results on the uptake of $^{35}SO_4^{2-}$ by tomato leaves and on the incorporation into TCA-insoluble fractions in a compatible and an incompatible combination are shown in Table 1. Both controls showed a higher uptake of $^{35}SO_4^{2-}$ (total radioactivity in the soluble protein fraction) than the corresponding inoculated leaves. However, the ratio of incorporation of $^{35}SO_4^{2-}$ into the TCA-insoluble fraction to the total uptake was about the same in the compatible interaction (Cf4/race 4) and the Cf4 control, but was almost 40% higher in the incompatible interaction (Cf5/race 4) compared with the Cf5 control. The uptake of [^{3}H(G)]L-amino acids by tomato leaves was less than 10% of the uptake of $^{35}SO_4^{2-}$, while incorporation (as dpm/µg protein) in the TCA-insoluble fraction was also lower (results not shown). $^{35}SO_4^{2-}$-labelled soluble proteins were subjected to polyacrylamide gel electrophoresis. After electrophoresis the gels were stained, scanned, sliced, and then the radioactivity in each slice was determined. Radioactivity of each slice was plotted in densitograms. Results are shown in Fig. 3. From densitometer tracings of electrophoretic patterns it is evident that protein band R_F 29 was present in the incompatible interaction (Cf5/race 4). There was no significant difference in distribution of radioactivity in the 4 gels in the overall protein bands occurring between R_F 10 and R_F 100, except for protein band R_F 29. In the incompatible interaction (Cf5/race 4) incorporation of $^{35}SO_4^{2-}$ in protein band R_F 29 was considerably higher than in the compatible interaction and in both controls. Inoculation of Cf4 plants with race 5 also resulted in enhanced incorporation of $^{35}SO_4^{2-}$ in protein band R_F 29. Increased radioactivity in protein band R_F 29 was due to incorporation of $^{35}SO_4^{2-}$ and not to non-specific binding of label, because electrophoresis of the soluble protein fraction of an incompatible interaction which had been incubated for 1 hour with $^{35}SO_4^{2-}$ followed by slicing the gel and counting the

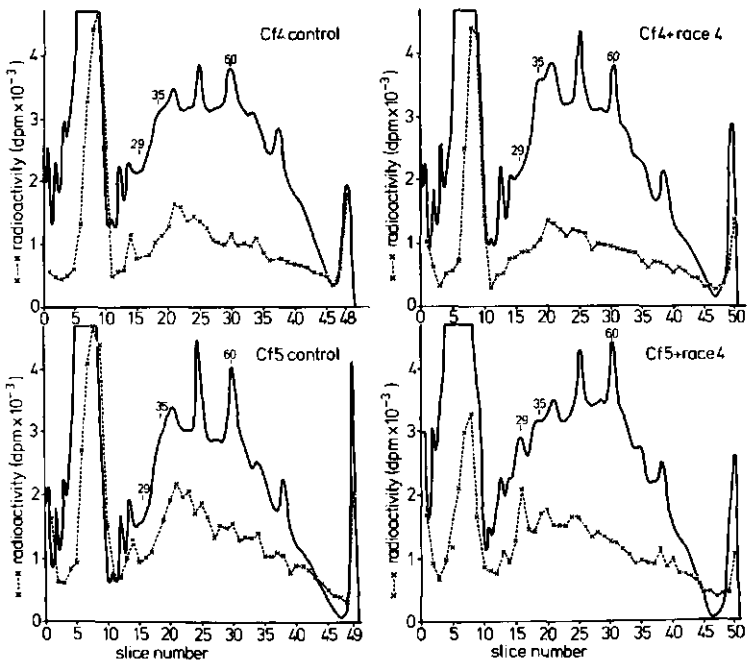
Table 1. Uptake of $^{35}SO_4^{2-}$ by tomato leaves and incorporation into the TCA-insoluble leaf protein fractions of near-isogenic lines Cf4 and Cf5, four days after inoculation with race 4 of *Cladosporium fulvum*[a)]

Fraction	Radioactivity (d.p.m. x 10^{-3} per 100 µl of fraction[b)])			
	Cf4 (control)	Cf4/race 4	Cf5 (control)	Cf5/race 4
Soluble leaf protein fraction (A)	581	495	607	402
TCA-insoluble leaf protein fraction (B)	34	29	47	43
B/A (%)	5.8	5.9	7.7	10.6

a) Data are the mean of duplicates from one experiment representative of two experiments.

b) One hundred µl fractions of all samples contained about 900 µg protein.

Fig. 3. Densitometer tracings of soluble leaf proteins of near-isogenic lines Cf4 and Cf5 (control leaflets) and leaflets four days after inoculation with race 4 of *Cladosporium fulvum*. Three days after inoculation compound leaves, with control and inoculated leaflets, were fed for one day with 25 µCi $^{35}SO_4^{2-}$ and then the soluble leaf proteins were subjected to electrophoresis on 7% polyacrylamide gels. The gels were stained, scanned at 620 nm (-) and sliced transversely in 1-mm slices. The radioactivity of each slice is indicated in the densitograms (*---*) as well as R_F 29 and R_F 35.



slices, did not show increased radioactivity in this band (results not presented). From these results it can be concluded that in tomato leaves the protein having R_F 29 was apparently synthezised *de novo* after inoculation with an avirulent race of *Cladosporium fulvum*.

Relation between the presence of protein band R_F 29 in tomato leaf and resistance to Cladosporium fulvum.

Since the appearance of protein band R_F 29 coincided with early fungal growth inhibition in resistant near-isogenic lines of tomato, it was investigated whether the protein having R_F 29 played a role in fungal growth inhibition. As shown in Plate 1Ac10, Bc10, Cc10, Dc10 protein band R_F 29 was present in control leaflets opposite to inoculated ones at ten days after inoculation. Ten days after inoculation of tomato leaflets with an avirulent race of *Cladosporium fulvum* the opposite control leaflets were inoculated with a virulent race and fungal growth was determined at various times after inoculation. Growth of virulent races in leaflets which contained protein band R_F 29 was not inhibited. Assuming that protein band R_F 29, present in these control leaflets opposite to inoculated ones is identical to protein band R_F 29 in inoculated leaves, it is unlikely that this protein, present in these concentration does render resistance of tomato leaves to *Cladosporium fulvum*.

Properties of protein band R_F 29.

Protein band R_F 29 showed a faint positive reaction with Schiff's stain for carbohydrates [27], so it is likely to be a glycoprotein. On SDS gels protein band R_F 29 sometimes gave more than one band, which made it difficult to determine its precise molecular weight. Estimations of molecular weight ranged between 55000 and 70000.

Changes in activities of various enzymes following inoculation with Cladosporium fulvum.

The changes in various enzyme activities resulting from fungal infection were investigated by histochemical staining procedures, to determine whether such changes might account for protein band R_F 29. Although, in tomato leaves, several enzymes increased in activity after inoculation with *Cladosporium fulvum*, significant quantitative changes occurred only in isoenzyme patterns of peroxidase. Plate 2 shows the distribution of peroxidase isoenzymes following electrophoresis of soluble protein fractions from healthy plants, compatible

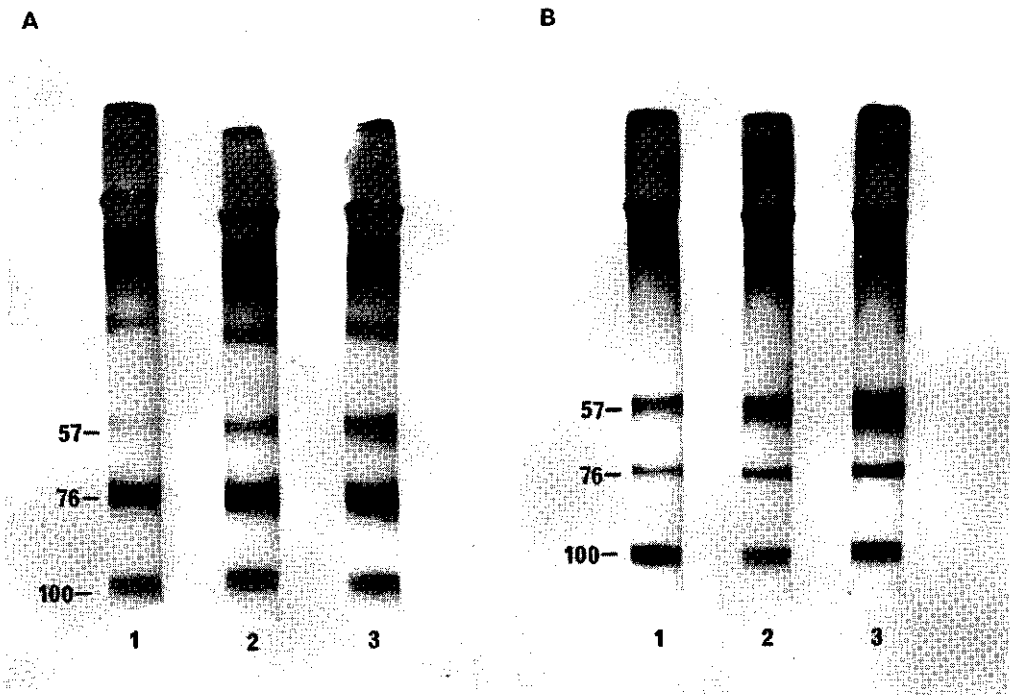


Plate 2. Electrophoretic patterns of isoperoxidases present in soluble leaf proteins of near-isogenic lines Cf4 and Cf5 on 7% polyacrylamide gels. A, gels incubated in 0.1 M potassium phosphate buffer, pH 6, containing 5 mM guaiacol and 5 mM H_2O_2; B, gels incubated in 0.1 M sodium citrate buffer, pH 2.5, containing 5 mM guaiacol and 5 mM H_2O_2. Each set of gels represents from left to right: 1, Cf4 control; 2, Cf4/race 4, four days after inoculation; 3, Cf5/race 4, four days after inoculation. Isoperoxidases, R_F 57 and R_F 76 are indicated.

and incompatible combinations on 7% polyacrylamide gels. A significant quantitative increase of isoenzymes R_F 57 and 76 occurred in the incompatible interactions and, though to a lesser extent, also in the compatible ones. Isoenzymes R_F 57 and 76 presumably correspond with the anionic tomato peroxidases C and B as described by Evans [8]. Protein band R_F 29 lacked peroxidase activity and also all other enzyme activities which were tested (see Materials and Methods).

DISCUSSION

In virus-plant interactions giving a hypersensitive response the appearance of several new proteins, additional to coat protein, has been reported [18, 21, 22]. It has been suggested that these new proteins were host-specific and related to the hypersensitive response [22].

Protein band R_F 29 occurring in tomato leaves after inoculation with *Cladosporium fulvum* is presumably also of host origin, because it appeared also in non-inoculated leaflets opposite to the inoculated ones. This suggests that the inducer of band R_F 29 is systemic, for it is unlikely that a protein with a molecular weight of 55000 - 70000 would be transported through the vein-system from the inoculated leaflet to the opposite control leaflet. Carpita *et al.* [1] showed that the limiting diameter of pores in the walls of living plant cells, through which molecules can freely pass, ranges from 35 - 52 Å. This means that only molecules up to a molecular weight of about 7000 can freely pass. In contrast to the systemically induced protein in virus-infected plants, which are thought to be associated with acquired systemic resistance [20, 22], mycelial growth of virulent races of *Cladosporium fulvum* was not inhibited in tomato leaves containing systemically induced protein band R_F 29. However, it still could be possible that the systemically induced protein band R_F 29 is immobilized soon after synthesis, which would hamper measuring its effect on fungal growth in the way we did. Systemically induced resistance, as in cucumber, watermelon and muskmelon against *Colletotrichum lagenarium* [2, 10] has not been observed in tomato plants against *Cladosporium fulvum*.

The function of protein band R_F 29 is not known yet. Its appearance was strongly associated with the hypersensitive response in incompatible interactions. In compatible interactions it appears two to three days later, at a time when host cells showed some necrosis. The protein still accumulated when the process of host cell necrosis was finished. This suggests that the appearance of protein band R_F 29 is more likely a consequence than a cause of the hypersensitive response. The fact that protein R_F 29 is already present in healthy plants,

even though in very low amounts, suggests that its accumulation is likely not be regarded as a specific response of the resistance gene Cf4 and Cf5 directed to race 4 and 5 of *Cladosporium fulvum*.

Protein band R_F 29 is unlikely to be a protein solubilized from plant tissue as a consequence of the hypersensitive response, because protein band R_F 29 was synthezised *de novo*. The induction and appearance of protein band R_F 29 showed some similarities with that of proteinase inhibitors in tomato plants which accumulate after wounding [23]. Preliminary experiments, however, showed that protein band R_F 29 did not have proteinase-inhibiting activity.

Recently Esquerré-Tugayé *et al.* [6, 7] reported evidence for the accumulation of hydroxyproline-rich glycoproteins in cell wall of diseased plants as a defense mechanism. In future research we are intending to study the similarities between these hydroxyproline-rich glycoproteins and protein band R_F 29.

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DIFFERENTIAL ACCUMULATION OF PHYTOALEXINS IN TOMATO LEAVES BUT NOT IN FRUITS AFTER INOCULATION WITH VIRULENT AND AVIRULENT RACES OF *CLADOSPORIUM FULVUM*

by

P.J.G.M. de Wit and W. Flach

ABSTRACT

Leaves of near-isogenic lines of tomato, carrying resistance gene Cf4 or Cf5 to *Cladosporium fulvum*, were both inoculated with race 4 and 5 of this fungus. Accumulation of two phytoalexins occurred more rapidly in the two incompatible combinations (Cf4 + race 5; Cf5 + race 4) than in the two compatible ones (Cf4 + race 4; Cf5 + race 5) in the whole leaf as well as in the lower epidermis. Hyphal growth in incompatible combinations was inhibited three to five days after inoculation, shortly after stomatal penetration. At this time, accumulation of phytoalexins was observed only in incompatible interactions. Following prolonged incubation phytoalexins also accumulated in compatible interactions, when there was already abundant fungal growth. The identity of the phytoalexins is not yet known.

Inoculation of pericarp tissue of tomato fruits of both near-isogenic lines with *Cladosporium fulvum* resulted in a very rapid accumulation of rishitin and four unidentified phytoalexins, two of which were identical to those occurring in tomato leaves with respect to their relative retention and R_F value. The concentration of rishitin, the main phytoalexin in tomato fruit, reached its maximum one to two days after inoculation and decreased later. In contrast to tomato leaves, in fruits no differential accumulation of phytoalexins in incompatible and compatible interactions was observed at an early stage of infection.

The phytoalexins in tomato leaves and fruits accumulated only after interaction with fungal material; they did not accumulate in wounded controls or after treatment with $HgCl_2$ and terramycin. For this reason, they cannot be regarded as general stress metabolites.

INTRODUCTION

In studies on the interaction of *Cladosporium fulvum* and tomato plants, carrying the Cf2, Cf4 or Cf5 genes for resistance to this pathogen, ultrastructural differences between compatible and incompatible combinations have been observed [4, 10, 11]. In incompatible interactions fungal growth was inhibited completely soon after stomatal penetration. The stomata and surrounding cells reacted by callose deposition and necrosis. Besides callose, which was deposited between plasma membrane and cell wall, extracellular material was present on the outer cell surface of mesophyll cells contacted by avirulent races of *Cladosporium fulvum* [4, 11]. The mechanism of fungal growth inhibition in incompatible interactions has not been elucidated.

In several other host-pathogen interactions accumulation of phytoalexins often occurred more rapidly and to a larger extent in incompatible interactions than in compatible ones [2, 21]. In these systems phytoalexins may play a role in the mechanism of resistance.

In tomato plants infected with *Cladosporium fulvum* phytoalexins have not been detected previously. The most important phytoalexins occurring in *Solanaceae* are sesquiterpenes [17]. Many members of this group, like rishitin, lubimin, phytuberin, rishitinol, hydroxylubimin, anhydro-β-rotunol and solavetivone have been isolated from potato tubers. The only identified terpenoidal compound so far reported from tomato fruits is rishitin [9, 15, 18].

The aim of the present study was to investigate whether the production of sesquiterpenoidal phytoalexins was induced in tomato leaves and fruits after inoculation with *Cladosporium fulvum* and whether differences occurred in accumulation of phytoalexins in compatible and incompatible combinations of tomato and *Cladosporium fulvum*. During this study near-isogenic lines of tomato, carrying the resistance gene Cf4 or Cf5, became available. For this reason we switched from tomato varieties carrying the resistance gene Cf2 or Cf4, used in previous studies [4], to near-isogenic lines. Preliminary results of this study have been published elsewhere [5].

MATERIALS AND METHODS

Plants.

Seeds of near-isogenic lines of Moneymaker, carrying resistance gene Cf4 (immune to race 5 and susceptible to race 4) or gene Cf5 (susceptible to race 5 and

immune to race 4) (generously supplied by Mrs Boukema, Institute for Horticultural Plant Breeding, Wageningen) were sown in trays with peat soil (Trio No. 17) and after two weeks seedlings were transplanted into pots (diam. 18 cm). Plants were kept in the glasshouse at temperatures between 20 and 28 ^{o}C and about 60% r.h. Light intensity was kept between 10000 and 15000 lx each day.

Fungi.

Monospore cultures of *Cladosporium fulvum* race 4 and 5 (kindly supplied by Mr Hubbeling, Research Institute for Plant Protection, Wageningen) were subcultured on potato dextrose agar (PDA) at 22 ^{o}C and stored at 4 ^{o}C. To ensure that races maintained their virulence, tomato plants were inoculated and the pathogen reisolated twice a year. Conidia from three-week-old cultures on PDA were used for inoculation. Conidia were harvested from plates and washed several times with distilled water by centrifugation. *Cladosporium cucumerinum*, from our laboratory, was subcultured also on PDA and used in thin-layer chromatography bioassays.

Inoculation.

Plants. The third, fourth or fifth leaf of six to seven-week-old tomato plants was inoculated on the abaxial side by spraying with a suspension of 10^{6} to 10^{7} conidia ml^{-1}. Plants were allowed to dry for 2 h and then sprayed twice again to provide a dense and homogeneous inoculum. Plants were incubated in a polyethylene-covered box. Optimal conditions for penetration were created by closing the box at night to stimulate conidial germination and hyphal growth, and by opening it in the morning to stimulate opening of the stomata, necessary for fungal penetration.

Fruits. Green tomato fruits with a diameter of 20 to 30 mm, from plants carrying the Cf4 or Cf5 gene for resistance, were used for inoculation. Since intact fruits are not susceptible to *Cladosporium fulvum* (due to lack of stomata) they were inoculated in the following ways. (1) Fruits were sterilized in 96% ethanol. A sterile conidial suspension of *Cladosporium fulvum* was injected with a syringe in the air space between locule tissue and pericarp. Injected fruits were incubated in a tray at 25 ^{o}C and a r.h. near 100%. (2) Fruits were sterilized in 96% ethanol and cut into four to eight pieces. Seeds with surrounding tissue were gently removed aseptically and the inner surface of the exposed

pericarp of each piece was inoculated with 100 µl of a conidial suspension of *Cladosporium fulvum*. Inoculated pieces were placed in sterile Petri dishes and incubated in the same way as described under (1).

Sampling methods.

In time-course experiments leaves were picked several days after inoculation and main veins were removed. Samples of 3 g (fresh wt) were taken and stored in a freezer at -20 °C. When accumulation of phytoalexins in the lower epidermis was studied, the latter was stripped off by a fine pair of tweezers and samples of 1 g (fresh wt) were taken. In experiments with fruit tissue, samples of 8 or 10 g (fresh wt) were taken.

Histological methods.

Histological examination was carried out as described previously [4]. In time-course experiments the extent of leaf colonization by *Cladosporium fulvum* was examined by light and fluorescence microscopy. Length of penetrated hyphae in the host was measured by a micrometer. After stomatal penetration the fungus initially grew with a straight hypha between epidermis and spongy mesophyll cells. At this stage the number of host cells contacting a penetration hypha was positively correlated with hyphal length.

Extraction of phytoalexins.

Frozen tissue samples of leaf, lower epidermis or fruit were homogenized in 10 ml methanol per g (fresh wt) with a "Virtis 45" mixer. After 1 to 2 h of extraction homogenates were filtered through a Büchner funnel. The filtrates were dried under reduced pressure at 40 °C and the residue was dissolved in 60% methanol, 5 ml per g fruit tissue or 15 ml per g leaf tissue. The methanol fraction was partitioned once with an equal volume of chloroform. This was sufficient to extract 95 to 97% of added rishitin. The chloroform extract was taken to dryness and the residue dissolved in a small volume of acetone (fraction I). The residual 60% methanol fraction was evaporated to dryness and taken up in a small volume of methanol (fraction II). The samples were stored overnight at -20 °C before they were subjected to thin-layer chromatography.

Thin-layer chromatography (t.l.c.).

Extracts from fruit or leaf tissue were applied on silicagel plates (Kieselgel

$60F_{254}$, 0.2 mm thickness, Merck) which were developed with $CHCl_3$: MeOH (95 : 5, v/v) (fraction I) or with $CHCl_3$: MeOH (3 : 1, v/v) (fraction II). After being dried the plates were sprayed with a solution of vanillin (3 g) in 100 ml ethanol which was acidified with 0.5 ml sulphuric acid, and heated to 120 °C for detection of sesquiterpenes [13]. Diazotized sulphanilic acid was used as a spray reagent for detection of phenols [16].

T.l.c. bioassays.

For detection of fungitoxic substances, developed thin-layer plates were dried and sprayed with a conidial suspension of *Cladosporium fulvum* or *Cladosporium cucumerinum* in B_5-medium [8] or in the medium described by Van Dijkman & Kaars Sijpesteijn [20], respectively. Sprayed plates were incubated in glass trays at 25 °C and a r.h. near 100%. Inhibition zones appeared two to three days after incubation. Identical results were obtained after spraying with *Cladosporium fulvum* and with *Cladosporium cucumerinum*. Inhibition zones caused by compounds present in chloroform extracts of tomato leaves were replicated on tracing paper (Schoellershammer Hochtransparent, No. 205 glatt, Düren, W. Germany), cut out and weighed. From these weights an accurate estimation of the size (mm^2) of the inhibition zones could be obtained. In similar bioassays amounts of methyl benzimidazol-2-yl carbamate (MBC), the active component of the systemic fungicide benomyl, could be accurately calculated using the regression equation $Y = a + b \ln x$, where a and b are constants, Y is the relative size of the inhibition zone and x is the amount of MBC [3]. Such a clear-cut relationship unfortunately was not found for components B and C present in chloroform extracts of tomato leaves, due to relative insensitivity of the test fungi to low concentrations of these compounds. This means that the inhibition zones are smaller than the actual spots of the fungitoxic compounds; at their margin the concentration is too low to inhibit growth of the test fungi. For this reason, data on the size of inhibition zones should be understood as being semi-quantitative only.

Gas-liquid chromatography (g.l.c.).

G.l.c. was carried out according to a modification of the method of Price *et al.* [13]. Zones of inhibition on t.l.c. plates, including rishitin, were cut out and eluted with 3 ml of acetone. The eluate was evaporated to dryness and methyl stearate or methyl arachidate dissolved in cyclohexane and ethyl acetate (1 : 1, v/v) was added as an internal standard. Samples of 3 µl were estimated on a Varian 3700 using a 152 cm x 4 mm i.d. glass column packed with

10% OV 210 on Diatomite CQ 100 to 120 mesh (Chrompack). Nitrogen was used as carrier gas at a flow rate of 40 ml min^{-1} and detection was by flame ionization. Operating temperatures were as follows: injection port 220 °C; detector 250 °C and column 160 to 200 °C. The average response ratio for rishitin with respect to methyl stearate was 0.65. Quantitative analysis was carried out by relating the areas of the peaks to that of the internal standard. Pure standards of rishitin, phytuberin, phytuberol and lubimin were supplied by Mr J.W. Henfling (Lexington, Kentucky) and anhydro-β-rotunol and solavetivone were given to us by Dr D.T. Coxon (Food Research Institute, Norwich). Of known amounts of rishitin, applied on t.l.c. plates, developed with $CHCl_3$: MeOH (95 : 5, v/v), sprayed with *Cladosporium cucumerinum* and incubated for two days, 95 to 97% could be recovered by g.l.c.

RESULTS

Accumulation of phytoalexins in tomato leaves.

In the chloroform extracts of non-inoculated tomato leaves virtually no fungitoxic substances were detected. However, after inoculation with *Cladosporium fulvum* significant amounts of fungitoxic substances were observed. Zones of inhibition with R_F values 0.47 to 0.50 (B) and 0.42 to 0.45 (C) were predominant (Plate 1). In Table 1 the results of a time-course experiment on fungal growth and phytoalexin accumulation are presented. The length of penetration hyphae inside the leaf is expressed in µm and amount of fungitoxic substances B and C in mm^2 of their inhibition zones. In both incompatible interactions inhibition of fungal growth was correlated with the presence of a high level of phytoalexins. Fungal growth was not inhibited in the two compatible interactions and phytoalexins were detected first when hyphae were already five to seven times longer than in incompatible interactions. Assuming that phytoalexins are produced after interaction between fungal hyphae and plant cells, we conclude that in incompatible interactions more phytoalexins are produced and by fewer cells than in compatible ones.

In fraction II of inoculated as well as of uninoculated leaves an inhibition zone appeared just above the origin on t.l.c. plates developed in $CHCl_3$: MeOH (3 : 1, v/v). The area of this inhibition zone did not change in compatible or incompatible interactions. It is improbable, therefore, that the substance involved is a phytoalexin.

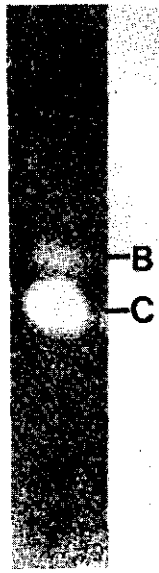

Plate 1. Inhibition zones (B and C) in t.l.c. bioassays of chloroform extracts from tomato leaf four days after inoculation with an avirulent race of *Cladosporium fulvum*. Amounts applied were equivalent to 3 g (fresh wt) of tomato leaf.

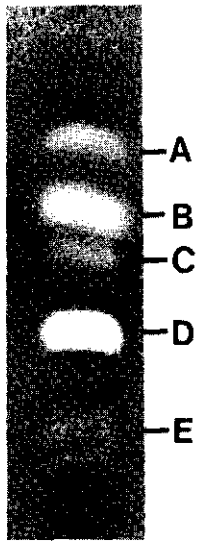

Plate 2. Zones of inhibition in t.l.c. bioassays of chloroform extracts from tomato fruit tissue four days after inoculation with *Cladosporium fulvum* (race 4 or 5). Zone D is rishitin, while zone A, B, C and E are unknown antifungal compounds of which B and C are identical to B and C in tomato leaf with respect to their relative retention and R_F value. Amounts applied were equivalent to 8 g (fresh wt) of tomato fruit tissue.

Table 1. Size of inhibition zones B and C in mm^2 on t.l.c. bioassays of chloroform extracts of tomato leaves[a] from two near-isogenic lines (Cf4 and Cf5) at various times after inoculation with virulent and avirulent races of *Cladosporium fulvum*[b]. At the same time, the length of the penetration hyphae was measured in µm[c].

Host-pathogen combination		Time after inoculation (h) 64	98	117
Cf4 (control)				
inhibition zones (mm^2)	B	0	0	0
	C	0	0	0
Cf5 (control)				
Inhibition zones (mm^2)	B	0	0	0
	C	0	0	0
Cf4 + race 4 (compatible)				
Inhibition zones (mm^2)	B	0	0	74
	C	0	0	120
Length penetration hyphae (µm)		14 ± 8	62 ± 20	116 ± 32
Cf4 + race 5 (incompatible)				
Inhibition zones (mm^2)	B	62	134	170
	C	80	192	260
Length penetration hyphae (µm)		8 ± 3	12 ± 3	19 ± 7
Cf5 + race 4 (incompatible)				
Inhibition zones (mm^2)	B	40	128	155
	C	80	244	266
Length penetration hyphae (µm)		8 ± 3	9 ± 3	20 ± 8
Cf5 + race 5 (compatible)				
Inhibition zones (mm^2)	B	0	0	50
	C	0	0	80
Length penetration hyphae (µm)		21 ± 9	80 ± 34	137 ± 26

a) 3 g (fresh wt) was extracted.
b) Data are from one experiment, representative of three separate experiments.
c) Values are the mean (± standard deviation) of 25 measurements.

Accumulation of phytoalexins in the lower epidermis.

Because fungal growth in incompatible interactions was strongly inhibited soon after stomatal penetration and the stomatal guard cells were the first to react after contact with an avirulent race [4] accumulation of phytoalexins in the lower epidermis of tomato leaves was studied. The lower epidermis was stripped off three and five days after inoculation and 1 g (fresh wt) was examined for the presence of phytoalexins. Results, shown in Table 2, were comparable with those shown for whole leaves. Inhibition zones were only present in incompatible interactions. Epidermal strips contained only a few mesophyll cells, as

Table 2. Size of inhibition zones B and C in mm^2 on t.l.c. bioassays of chloroform extracts of epidermal strips[a] from two near-isogenic lines (Cf4 and Cf5) at various times after inoculation with virulent and avirulent races of *Cladosporium fulvum*[b]. At the same time, the length of the penetration hyphae was measured in µm[c].

Host-pathogen combination		Time after inoculation (days) 3	5
Cf4 (control)			
Inhibition zones (mm^2)	B	0	0
	C	0	0
Cf5 (control)			
Inhibition zones (mm^2)	B	0	0
	C	0	0
Cf4 + race 4 (compatible)			
Inhibition zones (mm^2)	B	0	0
	C	0	0
Length penetration hyphae (µm)		45 ± 14	155 ± 35
Cf4 + race 5 (incompatible)			
Inhibition zones (mm^2)	B	103	130
	C	129	235
Length penetration hyphae (µm)		15 ± 10	37 ± 11
Cf5 + race 4 (incompatible)			
Inhibition zones (mm^2)	B	74	163
	C	235	260
Length penetration hyphae (µm)		25 ± 10	38 ± 14
Cf5 + race 5 (compatible)			
Inhibition zones (mm^2)	B	0	0
	C	0	0
Length penetration hyphae (µm)		35 ± 14	165 ± 38

a) 1 g (fresh wt) was extracted.
b) Data are from one experiment, representative of two separate experiments.
c) Values are the mean (± standard deviation) of 25 measurements.

appeared from electrophoresis patterns of the soluble protein fraction of these strips which showed only a minor band of fraction I protein, which is specific for chloroplasts. This indicates that a considerable amount of phytoalexins in incompatible interactions is produced by or accumulates in epidermal tissue. No phytoalexins were detected in epidermal tissue of both compatible interactions during the first five days after inoculation.

Accumulation of phytoalexins in tomato fruits.

Rishitin. Injection of conidial suspensions of both races of *Cladosporium*

fulvum in tomato fruits resulted in production of phytoalexins. In this case standardization of injections was difficult and it was impossible to get reproducible results with respect to amounts of accumulated rishitin. However, inoculation of pericarp tissue with conidial suspensions of both races of *Cladosporium fulvum* gave reproducible results.

In tomato fruits, inoculated with *Cladosporium fulvum*, rishitin could be detected 12 h after inoculation and reached its maximum two to three days after inoculation. In a time-course experiment amounts of rishitin were assessed by g.l.c. The results, presented in Table 3, do not show significant differences in amounts of rishitin in compatible (Cf4 + race 4; Cf5 + race 5) and incompatible (Cf4 + race 5; Cf5 + race 4) interactions. Rishitin appeared at the same time and at the same rate in both types of interactions. The concentration of rishitin decreased after four days, in some cases, to one-third of the maximum concentration, which was never greater than 5 $\mu g\ g^{-1}$ (fresh wt).

No significant differences in fungal growth on resistant and susceptible tomato fruit tissue could be observed by light and fluorescence microscopy. There was considerable mycelial growth in all combinations. Even *Cladosporium cucumerinum*, a non-pathogen of tomato, grew on tomato fruit tissue, while this fungus did not germinate on tomato leaves.

Although no differential accumulation of rishitin was observed in tomato fruit tissue after inoculation with virulent and avirulent races of *Cladosporium*

Table 3. Estimation of rishitin (µg rishitin per g fresh wt) in t.l.c. bioassays of chloroform extracts of fruits of near-isogenic lines of tomato (Cf4 and Cf5) at various times after inoculation with virulent and avirulent races of *Cladosporium fulvum* (2×10^6 conidia ml^{-1})[a].

Host-pathogen combination	Time after inoculation (h)				
	16	24	48	72	96
Cf4 (control)	0	0	0	0	0
Cf5 (control)	0	0	0	0	0
Cf4 + race 4 (compatible)	1	1	1	3	3
Cf4 + race 5 (incompatible)	1	2	4	3	1
Cf5 + race 4 (incompatible)	1	1	5	4	4
Cf5 + race 5 (compatible)	1	4	4	3	1

a) Data are from one experiment, representative of three separate experiments.

fulvum, induction of rishitin was not a result of wounding, as uninoculated, wounded pericarp tissue did not produce rishitin.

In another experiment tomato fruit tissue was inoculated with a conidial suspension of *Cladosporium fulvum* races 4 and 5, containing 5×10^5, 5×10^6 or 5×10^7 conidia ml^{-1}, a conidial suspension of *Cladosporium cucumerinum*, containing 5×10^6 conidia ml^{-1}, $HgCl_2$ (4×10^{-4} M, 2×10^{-3} M, 4×10^{-3} M or 10^{-2} M), terramycin (an oxytetracycline) (2×10^{-5} M, 10^{-4} M or 2×10^{-4} M) or H_2O respectively. Two days after inoculation samples were examined for the presence of rishitin. From the results shown in Table 4 it is clear that rishitin was only produced by tomato fruit tissue after interaction with fungal

Table 4. Estimation of rishitin in tomato fruit tissue two days after inoculation with *Cladosporium fulvum* or *Cladosporium cucumerinum*, or after treatment with different chemicals[a].

Treatment	Near-isogenic line	Amount of rishitin ($\mu g\ g^{-1}$ fresh wt)
(a) *Cladosporium fulvum*		
Race 5: 5×10^5 conidia ml^{-1}	Cf5	1
5×10^6 conidia ml^{-1}	Cf5	3
5×10^7 conidia ml^{-1}	Cf5	13
Race 4: 5×10^5 conidia ml^{-1}	Cf4	1
5×10^6 conidia ml^{-1}	Cf4	2
5×10^7 conidia ml^{-1}	Cf4	6
(b) *Cladosporium cucumerinum*		
5×10^6 conidia ml^{-1}	Cf5	3
$HgCl_2$		
4×10^{-4} M	Cf5	0
2×10^{-3} M	Cf5	0
4×10^{-3} M	Cf5	0
10^{-2} M	Cf5	0
Terramycin		
2×10^{-5} M	Cf5	0
10^{-4} M	Cf5	0
2×10^{-4} M	Cf5	0
H_2O		
Control	Cf5	0
	Cf4	0

a) Date are from one experiment, representative of two separate experiments.

material. The amount of rishitin produced was positively correlated with the concentration of the conidial suspension used for inoculation.

Other phytoalexins produced by tomato fruits. In tomato fruits inoculated with *Cladosporium fulvum* in addition to rishitin other fungitoxic compounds accumulated at a later stage of infection. Five zones of inhibition could be distinguished on t.l.c.-bioassays (Plate 2). Zone D (rishitin) appeared first, then zone B, and later zones A, C and E.

Comparison of phytoalexins occurring in tomato leaf and fruit.

Except for rishitin, the chemical nature of the phytoalexins occurring in tomato leaf and fruit tissues is not known. B and C in tomato leaf (Plate 1) are probably identical to B and C in tomato fruit (Plate 2) because their R_F values on t.l.c. and their relative retention on g.l.c. were the same (Table 5). In tomato leaves zone B is relatively small as compared with zone C; in tomato fruit the opposite is true. After spraying with vanillin in acidified ethanol zones B and C developed a grey-blue colour indicating that these phytoalexins might be sesquiterpenes. In addition, the phytoalexins in tomato leaf did not show a positive reaction for phenols after being sprayed with diazotized sulphanilic acid.

Table 5. R_F values on t.l.c. and relative retention on g.l.c. of fungitoxic compounds occurring in tomato leaf and fruit after inoculation with *Cladosporium fulvum*.

Compound in Leaf	Fruit	R_F value[a)]	Relative retention[b)]
-	A	0.59 - 0.62	-
B	B	0.47 - 0.50	1.66
C	C	0.42 - 0.45	3.66
-	D[c)]	0.29 - 0.33	0.63
-	E	0.18 - 0.22	-

a) Solvent $CHCl_3$: MeOH (95 : 5, v/v). Data were computed from five separate experiments.

b) Measured with respect to methyl stearate (conditions: column temperature 200 °C; detector temperature 250 °C; injector temperature 220 °C; N_2 flow rate 40 ml min^{-1}).

c) Compound D is rishitin.

DISCUSSION

Various authors searched for phytoalexins in tomato leaves after inoculation with plant pathogens. Van Dijkman & Kaars Sijpesteijn [19, 20] did not detect compounds, toxic to *Cladosporium fulvum*, after inoculation of tomato leaves with this pathogen. El-Sayed [6] reported the presence of rishitin in tomato leaf tissue after inoculation with *Phytophthora infestans*, an observation which we could not confirm (unpublished results). In addition, infection of tomato leaves with various fungi led to increased levels of often unspecified phenols [12] and a number of flavonoids [14]. In our experiments, a significant accumulation of fungitoxic compounds occurred after inoculation with *Cladosporium fulvum*. Tomato leaves inoculated with an avirulent race of *Cladosporium fulvum* always reacted by deposition of extracellular material on the wall of mesophyll cells contacted by fungal hyphae [4]. In other host-pathogen systems, a similar type of extracellular material has been reported [1, 7]. Lazarovits & Higgins, who also worked with *Cladosporium fulvum* on tomato, reported that this extracellular material contained polyphenols [10]. The fungitoxic compounds we isolated from inoculated tomato leaves all gave a negative reaction for phenols, but might be sesquiterpenes. However, their chromatographic properties differed from a number of known sesquiterpenes, isolated from potato tubers [17]. The identity of these phytoalexins is presently under investigation.

In the early stages of infection, accumulation of phytoalexins in tomato leaves was positively correlated with incompatibility between host and parasite. Later on, differences between compatible and incompatible interactions, with respect to accumulated phytoalexins, were less distinct. This suggests that phytoalexins accumulated more rapidly in incompatible interactions, or that induction of phytoalexins in the compatible interactions was suppressed. The accumulation of phytoalexins in incompatible interactions occurred at about the same time after inoculation as fungal growth inhibition, while in compatible interactions production of phytoalexins started only after there was abundant fungal growth. Time-course experiments with tomato leaf have indicated that the rate of phytoalexin accumulation was race-specific.

The only identified terpenoidal phytoalexin so far reported from tomato fruit is rishitin [9, 15, 18]. In tomato fruits inoculated with *Cladosporium fulvum* rishitin was also the most significant phytoalexin that accumulated. Rishitin accumulated at the same rate and to the same concentration in compatible and incompatible interactions. Rishitin was determined in the whole 5 to 7 mm thick pericarp tissue, which might explain the low levels of it [$\leq 5\ \mu g\ g^{-1}$

(fresh wt)]. However, if rishitin was determined in the 1 to 2 mm thick top layer of the pericarp tissue, yields of 20 to 25 μg g^{-1} (fresh wt) could be obtained, which were inhibitory to *Cladosporium fulvum in vitro* (unpublished results). In fruit tissue, rishitin accumulated but did not significantly affect fungal growth. Possibly the fungus released elicitors for rishitin production (unpublished results), but never came in contact with rishitin itself, because it grew on the surface of the pericarp tissue. In contrast to tomato leaves, in tomato fruit tissue accumulation of phytoalexins was not race-specific.

The phytoalexins in tomato leaves and fruits accumulated only after interaction with fungal material; they did not accumulate in wounded controls or after treatment with $HgCl_2$ and terramycin. For this reason, they cannot be regarded as general stress metabolites.

The identification of unknown phytoalexins accumulating in tomato leaves and fruits and the mechanism of their elicitation will be the subject of further studies.

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INDUCTION OF POLYACETYLENIC PHYTOALEXINS IN *LYCOPERSICON ESCULENTUM* AFTER INOCULATION WITH *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)

by

P.J.G.M. de Wit and E. Kodde

ABSTRACT

After inoculation with *Cladosporium fulvum* (syn. *Fulvia fulva*), the causal agent of tomato leaf mold, tomato fruits and leaves accumulate the polyacetylenic phytoalexins *cis*-heptadeca-1,9-diene-4,6-diyne-3,8-diol (falcarindiol) and probably *cis*-heptadeca-1,9-diene-4,6-diyne-3-ol (falcarinol); in addition fruits also accumulate the novel compound, *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol.

Maximal concentration of falcarindiol and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol were 5 - 10 μg g^{-1} fresh wt. The complete mycelial growth inhibition of *Cladosporium fulvum* and *Cladosporium cucumerinum in vitro* were about 6 and 18 μg ml^{-1} for falcarindiol and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol, respectively.

To our knowledge this is the first report on the occurrence of polyacetylenic phytoalexins in *Solanaceae*.

INTRODUCTION

Many sesquiterpenes have been found as phytoalexins in solanaceous species [14]. In addition to the sesquiterpene rishitin, occurring in tomato fruit, several other still unidentified antifungal compounds, all absent from healthy tissue, have been detected in extracts from tomato fruit and leaf after inoculation with *Cladosporium fulvum* [8]. The antifungal compounds occurred and accumulated more rapidly in incompatible than in compatible interactions of *Cladosporium fulvum* and tomato leaves [8]. In contrast to tomato leaves, in

fruits no differential accumulation of antifungal compounds in incompatible and compatible interactions was observed [8].

In this paper we describe the identification of three phytoalexins, two of them earlier indicated as B and C [8], which, as described in this paper, all appeared to be polyacetylenes, which had not been found before in solanaceous species, neither as normal constituents, nor as phytoalexins. The toxicity of two of these compounds against *Cladosporium fulvum* and *Cladosporium cucumerinum* is also determined.

MATERIALS AND METHODS

Inoculation.

Monospore cultures of *Cladosporium fulvum* were subcultured as described before [8]. The third, fourth or fifth leaf of six to seven-week-old tomato plants or green tomato fruits with a diameter of 20 - 30 mm were inoculated with an a-virulent race of *Cladosporium fulvum* and incubated as described earlier [8].

Extraction and purification of phytoalexins.

Two or five days after inoculation of tomato fruits or leaves, respectively, the inoculated tissues were harvested, weighed and stored at -20 °C until extraction. The tissues were homogenized in methanol (10 ml g^{-1} fresh wt). After two hours of extraction at room temperature homogenates were filtered and the filtrates were dried under reduced pressure at 40 °C; the residue was dissolved in 60% aqueous methanol (5 ml g^{-1} fresh wt) and partitioned twice with an equal volume of chloroform. The combined chloroform fractions were taken to dryness, the residue dissolved in a small volume of acetone and applied to silica-gel plates (Kieselgel 60 F254, 0.2 mm thickness, Merck) and sequentially developed in equilibrated tanks in the following solvent systems (I) methanol : chloroform (5 : 95 v/v); (II) cyclohexane : ethylacetate (1 : 1 v/v); (III) hexane : diethylether (1 : 1 v/v). Plates were examined under u.v. light with a peak emission at λ 254 nm and appropriate quenching bands were eluted with acetone. Alternatively, the plates were sprayed with the location agent 1% $KMnO_4$ in 2% Na_2CO_3 (aq.) which reacts with polyacetylenes to give a yellow spot against a violet background [6]. Due to their slightly hydrophobic character polyacetylenes could also be located by simply spraying the developed plates with water, in which they showed up as a white spot against a light grey background.

Identification of polyacetylenes.

Gas-liquid chromatography (g.l.c.) was performed on a Varian 3700 with a 152 cm x 4 mm i.d. glass column packed with 10% OV 210 or 1% OV 225 on Diatomite CQ 100 to 120 mesh (Chrompack) at 200 ^{o}C with N_2 as the carrier gas at 40 ml min^{-1}. Mass spectrometry of peaks appearing after g.l.c. (g.c.-m.s.) was performed on a Micromass - 700 F. High resolution mass spectra were obtained with an A.E.I. M.S. 902 mass spectrometer. ^{1}H nuclear magnetic resonance (n.m.r.) spectra were recorded on a Varian XL-100-15 spectrometer, equipped with a pulse unit and a 620L-16K on-line computer system using tetramethyl silane (t.m.s.) as internal standard and deuterochloroform as solvent (typical spectral parameters were as follows: spectral width 1000 Hz (0.25 Hz/point), acquisition time 4 s, pulse width 25 µs and number of transients 150-400). Infra red (i.r.) spectra were recorded on a Hitachi E.P.I. G3 with chloroform as the solvent. Ultra violet (u.v.) spectra were determined in methanol with a Beckman (model 25) spectrophotometer.

Acetylation of polyacetylenes.

Polyacetylenes (500 µg) were dissolved in 0.5 ml of acetic anhydride, containing a trace of pyridine, and incubated for three hours at room temperature. The reaction product was chromatographed in solvent system III.

T.l.c. bioassays.

T.l.c. bioassays were carried out as described before [8]. Developed silica-gel plates were sprayed with a conidial suspension of either *Cladosporium cucumerinum* or *Cladosporium fulvum* (10^6 conidia ml^{-1}). The plates were incubated for two or seven days, respectively, to locate zones of inhibition.

Determination of falcarindiol and cis*-tetradeca-6-ene-1,3-diyne-5,8-diol.*

Concentrations of falcarindiol and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol in extracts were determined after t.l.c. in solvent systems I, II and III by absorbance measurements in methanol at 245 and 241 nm, respectively.

Slide bioassays.

Conidia of *Cladosporium fulvum* or *Cladosporium cucumerinum* were suspended in a medium containing 20 g glucose, 2 g casamino acids, 2 mg $ZnCl_2$, 2 mg $MnSO_4.4H_2O$, 1.5 g $MgSO_4.7H_2O$, 0.11 g KCl per 1 of 2.5 mM potassium phosphate buffer, pH 6

(5.10^4 conidia ml^{-1}). One hundred µl of this suspension was pipetted onto glass microscope slides within a circle of vaseline. Samples (5 µl) of a series of concentrations of phytoalexins in 96% aqueous ethanol were added to each slide. A 100 µl drop of medium containing 5 µl of 96% aqueous ethanol served as control. The slides were incubated in Petri dishes lined with moist filter paper at 25 °C. Germination as well as germ tube and mycelial growth were assessed and the approximate ED_{50}-values were determined.

RESULTS

Identification of polyacetylenes.

T.l.c. bioassays of extracts of tomato leaves previously inoculated with an avirulent race of *Cladosporium fulvum* run in solvent system I showed at least two antifungal bands with R_F 0.48 and R_F 0.43, earlier indicated as B and C, respectively [8]. In addition to these two bands, in fruits a third band D [8] with R_F 0.31 occurred, which contained in addition to the sesquiterpene rishitin (confirmed by m.s., n.m.r. and i.r.) another antifungal compound. The bands showing antifungal activity were further treated with solvent systems II and III. The u.v. spectra of the compounds eluted from the bands showed absorptions at about λ_{max}^{MeOH} 230, 245, and 260 nm, the maxima being characteristic of compounds with conjugated triple bounds in polyacetylenes [3].

The u.v. spectrum of the antifungal fraction of band B, with R_F values of 0.48, 0.72 and 0.30 in solvent systems I, II and III, respectively, showed absorption maxima at λ_{max}^{MeOH} 233 (500), 245 (400), and 260 (240) nm. G.c.-m.s. spectra showed peaks of m/e 260 (M^+, 3.5%), m/e 242 (M^+-H_2O, 5%), m/e 175 ($M^+-C_6H_{13}$, 14%) and m/e 157 ($M^+-H_2O-C_6H_{13}$, 62%). High resolution mass spectrometry revealed a molecular ion of m/e 260.1768 with elemental composition $C_{17}H_{24}O_2$ (theoretical 260.1776). The acetylated compound showed a molecular ion m/e 344 indicating that the original compound was a diol. I.r. and n.m.r. spectral data of the compound were similar to those obtained for *cis*-heptadeca-1,9-diene-4,6-diyne-3,8-diol, falcarindiol (Fig. 1a) [3, 4]. Falcarindiol accumulated to concentrations of 5 - 10 µg g^{-1} fresh wt after inoculation of tomato fruits or leaves with an avirulent race of *Cladosporium fulvum*.

The antifungal fractions of band C with R_F values of 0.43, 0.63 and 0.21 in solvent system I, II and III, respectively, showed two major peaks as determined by g.l.c. One of these peaks obtained by g.c.-m.s. gave a molecular ion of m/e 244 (M^+, 30%) and one of m/e 159 ($M^+-C_6H_{13}$, 100%). This fragmentation

pattern was similar with literature values of *cis*-heptadeca-1,9-diene-4,6-diyne-3-ol, falcarinol (Fig. 1b) [3, 4, 15]. Because of the presence of the unidentified compound confirmation of the structure by using n.m.r. was not possible. However, u.v. and i.r. data of most pure samples were very similar to literature values [3, 4, 15].

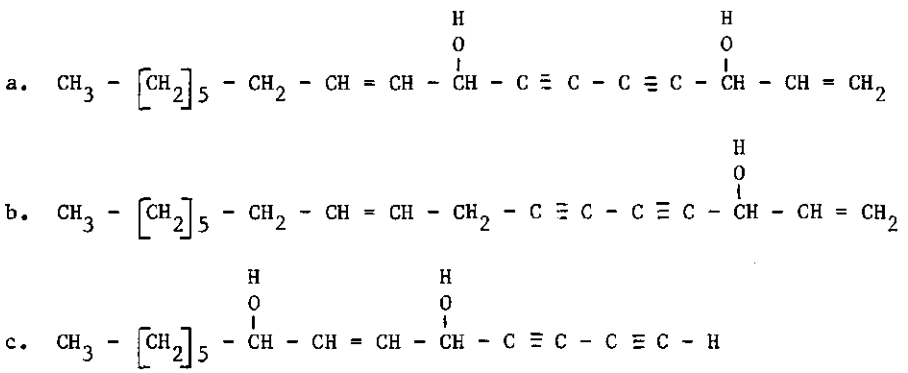
A third polyacetylenic phytoalexin could be isolated from band D [8] in solvent system I. This compound had R_F values of 0.31, 0.38 and 0.10 in solvent systems I, II and III, respectively. The u.v. spectrum showed absorptions at λ_{max}^{MeOH} 229 (400), 241 (400), 254 (225) and 284 (40) nm. G.c.-m.s. spectra showed peaks of m/e 220 (M^+, 3%), m/e 202 (M^+-H_2O, 5%) and m/e 117 ($M^+-H_2O-C_6H_{13}$, 100%). High resolution spectrometry revealed a molecular ion of m/e 220.1464 with elemental composition $C_{14}H_{20}O_2$ (theoretical 220.1463). The acetylated compound showed a molecular ion m/e 304 indicating that the original compound was a diol. The i.r. spectrum showed the presence of a terminal diyne group (3300 cm^{-1}) [5] and hydroxyl groups (3600, 3380, 1020 cm^{-1}). There was no absorbance at 960 cm^{-1} (absence of trans alkene). The n.m.r. spectrum was significantly different from that of falcarindiol [3, 4, 15]. It showed signals at δ ppm: 0.90 broad t (3H, CH_3-CH_2-); 1.30 broad s (8H, CH_3-$[CH_2]_4$-); 1.50 broad s (2H, -CH_2-CH_2-CH(OH)-); 2.20 broad s (1H, -C≡C-H), 2.85 broad s (2H, -CH(OH)-; twice); 4.45 broad s (1H, -CH_2-CH(OH)-CH=CH-); 5.30 broad m (1H, -CH=CH-CH(OH)-C≡C-); 5.63 broad m (2H, -CH=CH-).The complex multiplet at δ ppm: 5.90 occurring in falcarindiol was not present. In our case it was difficult with 1 mm n.m.r. tubes to get sharp absorptions. Most absorptions were a little broadened which hampered measurements of coupling constants.

The fragmentation pattern in the mass spectrum shows the typical allylic fission, indicating a terminal aliphatic group of C_6H_{13} [5]. The other terminal group is clearly a diyne as shown in the i.r. spectrum. These data together with the n.m.r. data only permit the structural formula as presented in Fig. 1c, *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol, a novel compound. This compound accumulated in inoculated tomato fruits to concentrations of about 10 μg g^{-1} fresh wt.

Fungitoxicity of polyacetylenes.

The fungitoxicity of falcarindiol and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol against *Cladosporium fulvum* and *Cladosporium cucumerinum* was determined. Falcarindiol showed approximate ED_{50}-values of 24 and 12 μg ml^{-1} for conidial

Fig. 1. Structural formulae of isolated polyacetylenes.
a. *cis*-Heptadeca-1,9-diene-4,6-diyne-3,8-diol (falcarindiol);
b. *cis*-Heptadeca-1,9-diene-4,6-diyne-3-ol (falcarinol);
c. *cis*-Tetradeca-6-ene-1,3-diyne-5,8-diol.



germination and germ tube elongation, respectively, which were not significantly different for both fungi. The effect of falcarindiol on mycelial growth of *Cladosporium cucumerinum* is shown in Plate 1A. The complete mycelial growth inhibition was at about 6 µg ml^{-1} for both fungi.

cis-Tetradeca-6-ene-1,3-diyne-5,8-diol was less fungitoxic than falcarindiol. The ED_{50}-values for conidial germination and germ tube elongation were 36 and 24 µg ml^{-1}, respectively. The effect of *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol on mycelial growth of *Cladosporium cucumerinum* is shown in Plate 1B. The complete mycelial growth inhibition was about 18 µg ml^{-1} for both fungi.

DISCUSSION

The polyacetylenic phytoalexins safynol [1] and dehydrosafynol [2] and the acetylenic keto furanoid phytoalexins wyerone [9], wyerone acid [13] and wyerone epoxide [11] have been detected in *Compositae* and *Papilionaceae*, respectively.

Falcarindiol and falcarinol have earlier been isolated from healthy tissue of *Falcaria vulgaris* [4, 5], *Daucus carota* [3, 10] and *Aegopodium podagraria* [15].

The production of falcarindiol, falcarinol and the novel compound, *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol in tomato upon infection with *Cladosporium fulvum*, is to our knowledge the first report on the occurrence of polyacetylenes as phytoalexins in *Solanaceae*.

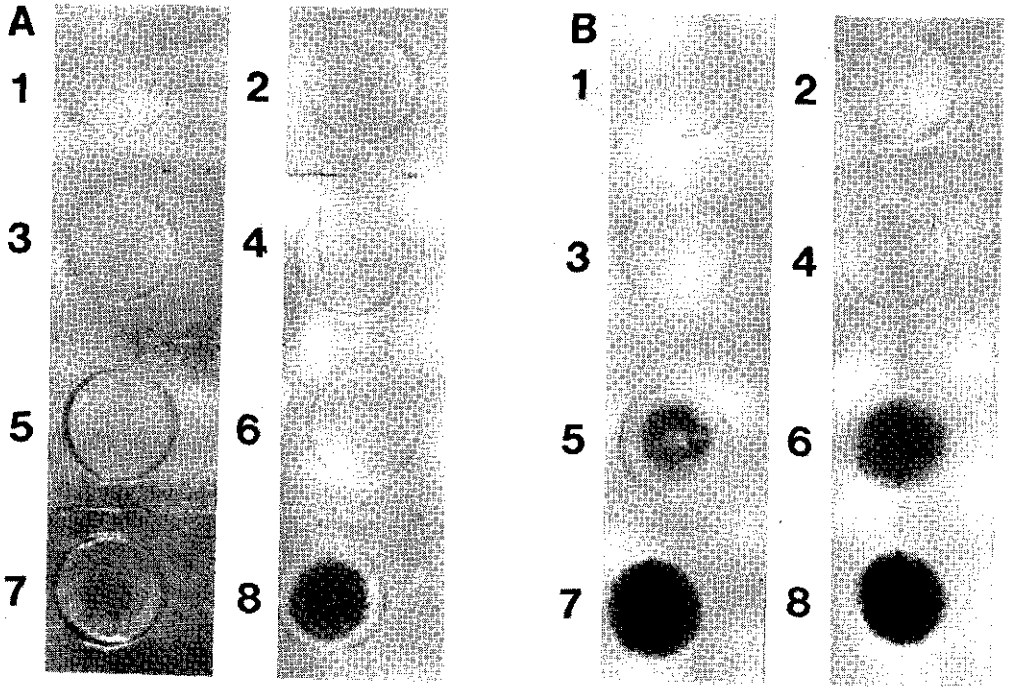


Plate 1. Effect of *cis*-heptadeca-1,9-diene-4,6-diyne-3,8-diol (A) and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol (B) on mycelial growth of *Cladosporium cucumerinum*. Concentration in growth medium: 1, 2, 3, 4, 5, 6, 7 and 8 containing 96, 72, 48, 36, 24, 12, 6 and 0 µg ml^{-1}, respectively.

As reported earlier [8], in tomato leaves phytoalexins accumulated earlier and often to a higher level in incompatible than in compatible interactions. The level to which falcarindiol accumulated nearly reached the ED_{50}-value of hyphal and mycelial growth *in vitro*. The concentration in the infected tissues might be higher. Kemp [13] found complete growth inhibition of *Cladosporium cucumerinum in vitro* by 10 µg ml^{-1} falcarindiol, which is in fairly good agreement with our findings. Fraction C, the most important band with antifungal activity occurring in tomato leaf [8] contained next to falcarinol at least one other phytoalexin. Identification of this latter compound was hampered due to thermal instability and photodecomposition, a phenomenon typical of polyacetylenes. In general it was much easier to purify the polyacetylenes from inoculated fruit tissue than from leaf tissue. The purification of the polyacetylenes needed the sequential development in three solvent systems and led to losses of often 30 - 40% per run. This interfered with exact quantification of the polyacetylenes.

This paper shows that *Solanaceae* are capable of producing different types of phytoalexins, a phenomenon earlier reported for *Leguminosae* which produce pterocarpanoid [12] as well as acetylenic phytoalexins [9, 11, 13].

Evidently, the distribution of polyacetylenic compounds does not reflect such strong taxonomic relationships among higher plants as suggested by Dahlgren [7].

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ISOLATION, PARTIAL CHARACTERIZATION AND SPECIFICITY OF GLYCOPROTEIN ELICITORS FROM CULTURE FILTRATES, MYCELIUM AND CELL WALLS OF *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)

by

P.J.G.M. de Wit and P.H.M. Roseboom

ABSTRACT

Culture filtrates, mycelium extracts and cell walls of *Cladosporium fulvum* (syn. *Fulvia fulva*) contained high molecular weight components which elicited rishitin accumulation in tomato fruit tissue. Elicitors from mycelium extracts (MEE) and cell walls (CWE) appeared to be five to ten times as active as those isolated from culture filtrates (CFE).

Elicitors isolated from two races of *Cladosporium fulvum* were not host-specific. Of the four non-hosts of *Cladosporium fulvum* which were assayed for phytoalexin accumulation after treatment with elicitor, pea and soybean reacted by accumulation of pisatin and glyceollin, respectively, while under the conditions used potato and jack bean did not accumulate rishitin and medicarpin, respectively.

The rishitin-inducing activity of CFE, MEE and CWE was sensitive to $NaIO_4$, α-mannosidase, Pronase, Proteinase K and also to NaOH, which suggest that the elicitors are glycoproteins. The rishitin-inducing activity of CFE was not bound to DEAE-Sephadex and could be purified threefold by chromatography on Con A-Sepharose 4B.

CFE contained mainly glucose and some mannose, galactose and protein. MEE and CWE contained five to ten times more mannose, galactose and protein than CFE. The protein part of all elicitors was especially rich in asparagine/aspartic acid, threonine, serine, glutamine/glutamic acid and proline.

INTRODUCTION

Phytoalexin production is often associated with a plant defense reaction [8, 9, 20, 29, 45, 47, 48]. Plants or plant parts treated with components of fungal origin are usually able to elicit the synthesis of the same phytoalexins as the pathogens themselves [2, 28]. These fungal compounds, termed elicitors by Keen [27], are reported to be a small peptide in the case of *Monilinia fructicola* [13] proteinaceous factors in the case of *Fusarium solani* [14] and *Botrytis cinerea* [17], a protein or glycoprotein in the case of *Rhizopus stolonifer* [41] and a polysaccharide or oligosaccharide in the case of *Colletotrichum* species [3, 4] and *Phytophthora megasperma* var. *sojae* [2, 5, 6, 7], respectively.

Lazarovits & Higgins [30] and Lazarovits *et al.* [31] have recently reported the specificity, purification and partial characterization of a glycoprotein toxin, produced by *Cladosporium fulvum* that caused callose formation, host cell necrosis and ion leakage in tomato leaf tissue. The toxin was neither race nor cultivar-specific; it had similar effects on all tomato cultivars whether or not a gene for resistance was present. Non-hosts of *Cladosporium fulvum* were not affected by the toxin.

In the present paper we report the isolation and partial characterization of glycoprotein elicitors from culture filtrates, mycelium and cell walls of two races of *Cladosporium fulvum* (syn. *Fulvia fulva*). Also we have studied their ability to induce the acummulation of rishitin in tomato fruit tissue and potato tuber slices, pisatin in pea pods, glyceollin in soybean cotyledons and medicarpin in Jack bean cotyledons. The possible rôle of these glycoprotein elicitors in the interaction between *Cladosporium fulvum* and tomato will be published elsewhere.

MATERIALS AND METHODS

Fungi, and preparation of elicitors from culture filtrates, cell walls and mycelium.

Monospore cultures of *Cladosporium fulvum*, race 4 and 5, were subcultured as described elsewhere [15]. Shake cultures were initiated by adding 10^7 conidia from two-week-old cultures grown on potato dextrose agar (PDA), to 250 ml Erlenmeyers containing 50 or 100 ml of B5-medium [23] or the medium described by Van Dijkman & Kaars Sijpesteijn [46]. The cultures were incubated for varying times in the dark at 23 ^{o}C on an orbital shaker (180 rev min^{-1}).

Cell-free culture filtrates were obtained by filtering the cultures twice through filter paper on a Büchner funnel. The filtrates were concentrated 20-fold under reduced pressure at 40 °C in a rotary evaporator and dialyzed against distilled water for 48 hours at 5 °C with four changes. After dialysis, any insoluble material was removed by centrifugation at 40000 g for one hour; the clear supernatant fluids were freeze-dried and stored at -20 °C. This lyophilized non-dialyzable fraction will be called culture filtrate elicitor (CFE). Elicitors of mycelial cell walls (CWE) of twelve-day-old cultures of both races of *Cladosporium fulvum* were prepared by the method described by Ayers *et al.* [6] and mycelial extracts by that of Lloyd [35]. In the latter method, mycelium was washed with water and then with acetone; the acetone-dried powder was extracted with 0.05 M potassium phosphate buffer, pH 7.0 (20 ml g^{-1}) by stirring at 100 °C for two hours. After centrifuging at 20000 g for ten min the residue was extracted twice more with 0.05 M potassium phosphate buffer, pH 7. Sodium acetate (18 mg ml^{-1}) and ethanol (three volumes) were added to the combined extracts and incubated overnight. Then the precipitate formed was centrifuged, dissolved in water and dialyzed extensively against distilled water. After dialysis any remaining insoluble material was removed by centrifugation at 40000 g for one hour and the supernatant freeze-dried. This freeze-dried material will be called mycelium extract elicitor (MEE). Fractionation, as described by Lloyd [35] was not carried out.

Bioassays of elicitors.

Tomato fruit tissue. In this bioassay fruits of the cultivar Sonato were used. These fruits (generously supplied by Mr J. Verburg, Department of Plant Physiology, Agricultural University, Wageningen) were available throughout the season. Fruits were disinfected in 96% ethanol and cut into eight pieces; the seeds and the surrounding tissue were carefully removed. One hundred µl of an aqueous elicitor solution was applied to the inner surface of each piece of pericarp tissue. The eight pieces used in each treatment were placed in 150 mm diameter Petri dishes, incubated in the dark at 25 °C for 48 hours, and then examined for the presence of rishitin.

Potato tuber tissue. The assay described by Tomiyama *et al.* [45] was used. Potato tubers, cv. Bintje, were disinfected in 0.3% H_2O_2 and cut into 15 mm thick discs with a diameter of approximately 40 mm. These were placed on moist filter paper in 150 mm diameter Petri dishes. Five hundred µl of an aqueous

elicitor solution was applied to the cut surface of each disc. After incubation for 48 hours at 25 °C in the dark, the 2 mm thick top-layer of the treated discs was examined for the presence of rishitin.

Pea pods. The assay described by Cruickshank & Perrin [12] was used. Halves of pea pods of two to three cm length, from plants grown in a growth chamber at 20 °C, were placed in 150 mm diameter Petri dishes. Two hundred µl of an aqueous elicitor solution was applied to the endocarp of each pod. The pods were incubated for 48 hours at 25 °C in the dark and then examined for the presence of pisatin.

Soybean cotyledons. According to the assay of Frank & Paxton [22] cotyledons detached from eight-day-old seedlings of soybean, cv. Harosoy 63, were sterilized for one hour in 0.3% H_2O_2 and then washed thoroughly with distilled water. A section of about one mm thick and six mm in diameter was cut from the abaxial surface of each cotyledon. The cotyledons were then placed on moist filter paper in 100 mm diameter Petri dishes and 20 µl of an aqueous elicitor solution was applied to the cut surface of each cotyledon. The cotyledons were incubated for 24 hours at 25 °C in the dark and then examined for the presence of glyceollin.

Jack bean cotyledons. Cotyledons detached from twelve-day-old seedlings of an unknown Jack bean cultivar were cut longitudinally, and placed on moist filter paper in 150 mm diameter Petri dishes. Two hundred µl of an aqueous elicitor solution was applied to the cut surface of each part of cotyledon. The cotyledons were incubated for 48 hours at 25 °C in the dark and then examined for the presence of medicarpin.

Extraction and estimation of rishitin, pisatin, glyceollin and medicarpin.

Rishitin. Rishitin was extraced using a slight modification of a method described before [15]. Extracts were fractionated using thin layer chromatography (t.l.c.) with $CHCl_3$: MeOH (95 : 5, v/v) as solvent. Due to their slightly hydrophobic character the zones containing rishitin could be localized on silica gel plates simply by spraying with water (pure rishitin was used as a reference). The zones were scraped off and eluted with three ml of acetone. The eluate was evaporated to dryness and then analyzed by gas chromatography (g.l.c.). Methyl stearate, dissolved in cyclohexane and ethyl acetate (1 : 1, v/v), was added as an internal standard. Samples of three µl were estimated on a Varian 3700 gas chromatograph

using a 152 cm x 4 mm i.d. glass column packed with 10% OV 210 on diatomite CQ 100-120 mesh (Chrompack). Nitrogen was used as the carrier gas at a flow rate of 40 ml min^{-1} and detection was by flame ionization. Operating temperatures were as follows: injection port 220 °C; detector 250 °C and column 200 °C. The average response ratio of rishitin with respect to methyl stearate was 0.65. Quantitative analysis was carried out by relating the area of each peak to that of the internal standard.

Pisatin. Pisatin was estimated by a slightly modified version of the method described by Cruickshank & Perrin [12]. Pea pods were homogenized in H_2O (five ml g^{-1}) and the homogenate was then extraced three times with an equal volume of light petroleum (b.p. 40 to 60 °C). The combined light petroleum extracts were evaporated to dryness, dissolved in a small volume of ethanol and applied to silica gel plates (Kieselgel 60 F254, 0.2 mm thickness, Merck), which were then developed with $CHCl_3$: MeOH (97 : 3, v/v). The area containing pisatin was located under ultra-violet light (λ_{max} 254 nm), scraped off and eluted with three ml of 96% ethanol. The concentration of pisatin was calculated from the measured O.D. at 309 nm, using an O.D. value of 1.00 as equivalent to 43.8 µg ml^{-1} [12].

Glyceollin. Glyceollin was extracted according to the method of Paxton (pers. comm.). Ten soybean cotyledons were extracted with five ml 60% ethanol in 0.01 M potassium phosphate buffer, pH 7.8, for 24 hours at 5 °C. The ethanol extract was partioned once with five ml chloroform. The chloroform phase was evaporated to dryness and the residue dissolved in ethanol. The concentration of glyceollin in ethanol was calculated from the measured O.D. at 285 nm, using an O.D. value of 1.00 as equivalent to 32.8 µg ml^{-1} [5].

Medicarpin. Medicarpin was extracted using a modified version of the method described by Higgins [25]. The treated Jack bean cotyledons (± 20 g) were homogenized in 96% ethanol (2.5 ml g^{-1}) and extracted three times with 96% ethanol. The combined fractions were concentrated under reduced pressure at 40 °C to about 20 ml, adjusted to pH 3 with 1 N HCl and extracted three times with an equal volume of chloroform. The combined chloroform fractions were concentrated under reduced pressure at 40 °C to about 20 ml and extracted three times with an equal volume of 0.2 N NaOH. The combined NaOH fractions were adjusted to pH 3 with 4 N HCl and extracted three times with an equal volume of chloroform.

The combined chloroform fractions were concentrated, applied to silica gel plates (Kieselgel 60 F254 0.2 mm thickness, Merck) and developed in hexane : ethyl acetate : methanol 60 : 40 : 1 (v/v/v). The area containing medicarpin was located under ultra-violet light (λ_{max} 254 nm), scraped off and eluted with three ml of 96% ethanol. Since the medicarpin samples contained some maackiain, the amount of medicarpin in these solutions was determined using the formula:

$$E_{287\ nm} - \frac{5128}{8511} \times E_{311\ nm} = 1$$

as equivalent to 34 µg ml^{-1} (ε_M = 7943 at 287 nm for medicarpin; ε_M = 5128 at 287 nm and ε_M = 8511 at 311 nm for maackiain) [20].

Testing the heat and pH stability, and sensitivity to $NaIO_4$ and enzymatic breakdown of elicitors.

Concentrations of all elicitor solutions were standardized to 1.0, 0.5 or 0.1 mg glucose quivalents per ml (anthrone assay [16]). Stability to heat was tested by boiling for ten min at 100 °C. To test the pH stability the elicitor solutions were treated for one hour or 22 hours with 0.01 N NaOH or 0.01 N HCl at 25 °C, neutralized with 1 N HCl or 1 N NaOH, respectively, and buffered with 10 µl of 1 M potassium phosphate buffer, pH 7. Periodate sensitivity was examined by treating elicitor solutions with 0.02 M $NaIO_4$ for 22 hours at 5 °C in the dark. To remove the excess of $NaIO_4$ the samples were dialyzed against water for 24 hours with four changes. Sensitivity to enzymatic breakdown was tested using the following enzymes, under conditions as indicated: Pronase (0.2 mg ml^{-1}, Calbiochem AG) and Proteinase K (0.2 mg ml^{-1}, Boehringer) in 0.25 M potassium phosphate buffer, pH 7.8, for 22 hours at 37 °C; Ribonuclease A (0.2 mg ml^{-1}, Sigma) in 0.25 M potassium phosphate buffer, pH 7, for 22 hours at 25 °C, α-glucosidase (0.2 mg ml^{-1}, Sigma) and β-glucosidase (0.2 mg ml^{-1}, Sigma) in 0.25 M potassium acetate buffer, pH 4.7, for 22 hours at 25 °C, α-galactosidase (0.2 mg ml^{-1}, Sigma) in 0.25 M potassium phosphate buffer, pH 5.8, for 22 hours at 25 °C; β-galactosidase (0.2 mg ml^{-1}, Sigma) in 0.05 M potassium phosphate buffer, pH 7.5, for 22 hours at 25 °C; α-mannosidase (0.2 mg ml^{-1}, Boehringer) in 0.025 M potassium acetate buffer, pH 4.5, for 22 hours at 25 °C. After enzyme treatments the samples were neutralized, if necessary, with 1 N NaOH or 1 N HCl and buffered with 10 µl of 1 M potassium phosphate buffer, pH 7.

Because the phytoalexin-inducing activity of the elicitors appeared to be

heat-stable all samples were heated for 10 min at 100 °C before being tested to sterilize the samples and to destroy activity of any enzyme added. Untreated elicitors added to 0.01 N NaCl, buffered with 10 µl 1 M potassium phosphate buffer, pH 7, served as control for the NaOH and HCl treatment, while extensively dialyzed 0.02 M $NaIO_4$ buffered with 10 µl 1 M potassium phosphate buffer, pH 7, served as control for the $NaIO_4$ treatment. Untreated elicitors added to heated enzyme solutions, buffered with 10 µl potassium phosphate buffer, pH 7, served as controls for the enzyme treatments. If any treatment partly or completely destroyed the phytoalexin-inducing activity of the elititor, then an extra control was added to test the effect of the partly or completely destroyed elicitor on the phytoalexin-inducing activity of the untreated elicitor. To test this, treated elicitors were added to known amounts of untreated elicitors and the phytoalexin-inducing activity of this mixture was tested.

Determination of phosphorus, carbohydrate and protein.

Phosphorus was determined by the method of Chen *et al.* [11]. Solutions were assayed for hexoses by the anthrone method [16] with D-glucose as a standard. Protein was determined by the method of Lowry *et al.* [37], as modified by Hartree [24], using bovine serum albumin as a standard.

Assay of monosaccharide and amino acid composition of elicitor preparations.

Aliquots of elicitor preparations containing one to two mg equivalents of glucose were hydrolyzed and the alditol acetates prepared and analyzed by the method of Jones & Albersheim [26].

Amino acids in the samples were estimated on an amino acid analyzer (Biotronic, LC 6000 E, coupled with an integrator, Spectra physics, system I) after hydrolysis in 6 N HCl at 110 °C for 24 hours.

Column chromatography.

Sephadex G-200, DEAE-Sephadex A-50 and Concanavalin A covalently linked to Sepharose 4B were obtained from Pharmacia. The equilibration and elution buffers, and column dimensions are described in the text.

RESULTS

Mycelial growth, extracellular production of glycoproteins and rishitin-inducing activity throughout the growth cycle of races 4 and 5 of Cladosporium fulvum.

Mycelial growth rates (based on mycelial dry wt) in B5 medium [23] were four to five times higher than in the medium used by Van Dijkman & Kaars Sijpesteijn [46]. The B5 medium was used throughout this study. Figure 1 shows the increase of mycelium dry wt and the production of carbohydrate and protein throughout the growth cycle of races 4 and 5 of *Cladosporium fulvum*. The production of glycoproteins (for evidence that these componenets are glycoproteins see Fig. 4) mainly occurred in the post-exponential phase. Protein measurements in samples obtained after ten and twelve days incubation were fairly inaccurate since cladofulvine [44] in the culture filtrate interfered with protein determination. After incubation for 2, 4, 6, 8, 10 or 12 days, fifty ml samples of culture fluid from both cultures were concentrated to 25 ml and dialyzed exhaustively. The dialyzed culture filtrates were tested for rishitin-inducing activity. Results are shown in Fig. 2. The culture filtrates of both races showed maximum rishitin-inducing activity after incubation for ten days. The ability of both races to induce rishitin synthesis was not significantly different.

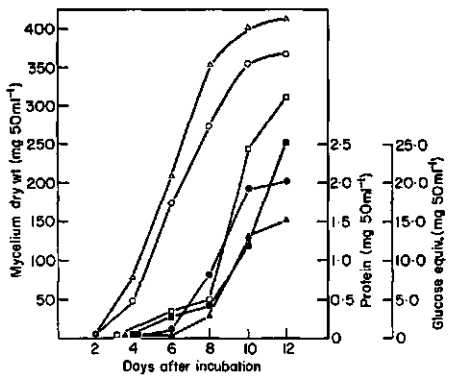

Fig. 1. Mycelial growth (○ race 4, △ race 5) extracellular production of protein (■ race 4, □ race 5) and carbohydrate (as glucose equivalents, ● race 4, ▲ race 5) throughout the growth cycle of races 4 and 5 of *Cladosporium fulvum*.

In later experiments dialyzed culture filtrates from cultures which had been incubated for ten days were used for the isolation of culture filtrate elicitor (CFE).

Relation between amount of CFE applied to tomato fruit tissue and rishitin produced.

Results on the response of tomato fruit tissue to varying amounts of CFE are presented in Fig. 3. Elicitors of both races induced similar quantities of rishitin. The dose response curve showed saturation by applying 100 to 200 µg of elicitor (in µg glucose equivalents) per g tomato fruit tissue.

Heat and pH stability, and sensitivity to $NaIO_4$ and various enzymes of rishitin-inducing activity of CFE.

In Table 1 results on heat and pH stability, as well as sensitivity to $NaIO_4$, α-mannosidase, Pronase and Proteinase K of rishitin-inducing activity of CFE of race 4 and 5 are presented. The rishitin-inducing activity of CFE from both races was heat stable and fairly pH stable. However, at high pH there was a significant decrease in rishitin-inducing activity after 22 hours of incubation. The rishitin-inducing activity was completely destroyed upon treatment with 0.02 M $NaIO_4$. Pronase and Proteinase K destroyed most of the rishitin-inducing activity of CFE, but RN-ase, α- and β-glucosidase and α- and β-galactosidase

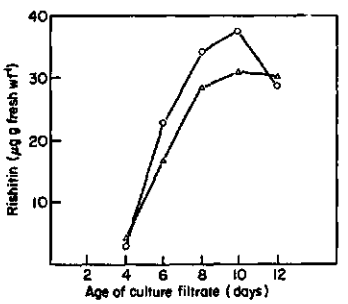

Fig. 2. The relation between the age of culture filtrates of race 4 (O) and race 5 (Δ) of *Cladosporium fulvum* and rishitin-inducing activity (µg rishitin per g fresh wt) in tomato fruit tissue.

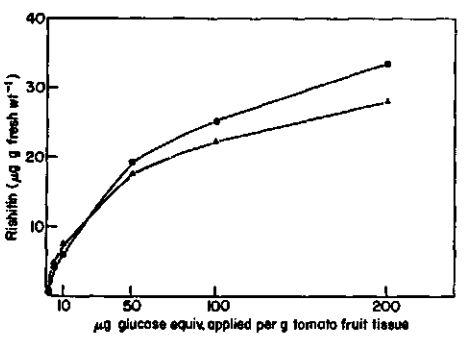

Fig. 3. The relation between amount of culture filtrate elicitor (CFE) (in µg glucose equivalents per g) applied to tomato fruit tissue and the amount of rishitin (µg g fresh wt^{-1}) produced: ●, CFE race 4; ▲, CFE race 5.

Table 1. Heat and pH stability, and sensitivity to $NaIO_4$ and various enzymes of rishitin-inducing activity of elicitors from culture filtrates (CFE)[a] of races 4 and 5 of *Cladosporium fulvum*.

Treatment[b] of CFE	Rishitin production as %[c] of CFE control[d]	
	Race 4	Race 5
Control	100	100
10 min 100 °C	97	103
pH 12; 0.01 N NaOH (1 h)	83	84
pH 12; 0.01 N NaOH (22 h)	17	22
pH 2; 0.01 N HCl (1 h)	97	96
pH 2; 0.01 N HCl (22 h)	57	48
0.02 M $NaIO_4$ (22 h)	0	0
α-mannosidase, 0.2 mg ml^{-1} (22 h)	41	32
Pronase, 0.2 mg ml^{-1} (22 h)	9	16
Proteinase K, 0.2 mg ml^{-1} (22 h)	21	26

a) The CFE concentration was 1 mg glucose equivalents per ml.
b) For treatments see Materials and Methods.
c) Data are the mean of duplicates from one experiment representative of a total of three experiments.
d) The absolute values of rishitin production for the CFE controls were 34 and 29 µg rishitin per g for races 4 and 5, respectively. For each treatment two other controls were included: 1) the various chemicals and heated enzymes alone or added to untreated CFE; they did not show any rishitin-inducing activity; 2) treated CFE added to untreated ones; they did neither inhibit nor increase rishitin-inducing activity.

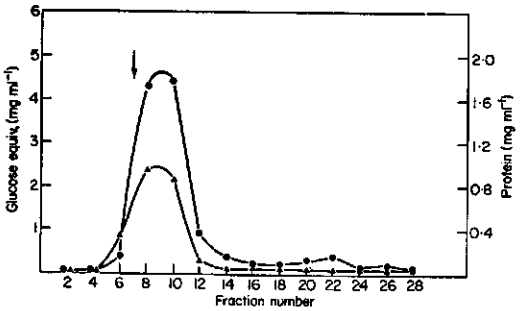

Fig. 4. Chromatography of culture filtrate elicitor (CFE) of race 4 of *Cladosporium fulvum* on Sephadex G-200. Twenty mg of CFE dissolved in 1 ml H_2O was applied to a column (1.6 x 30 cm) and fractions of 1.25 ml were collected. The arrow indicates the void volume. ●, Glucose equiv. (mg ml^{-1}); ▲, protein (mg ml^{-1}).

had no effect (results of latter not presented).

The rishitin-inducing activity of untreated CFE added to CFE, that was partly or completely destroyed by treating with NaOH, $NaIO_4$, α-mannosidase, Pronase and Proteinase K, was not significantly inhibited. This means that the decrease in rishitin-inducing activity of CFE after treatment with NaOH, $NaIO_4$, α-mannosidase, Pronase and Proteinase K was not due to artefacts. The loss of rishitin-inducing ability of CFE after treatment with $NaIO_4$, and the partial destruction after treatment with α-mannosidase, suggests that the carbohydrate part of CFE plays a role in rishitin induction. However, $NaIO_4$ treatment can also oxidize cysteine, methionine, tryptophan, proline, hydroxyproline, and NH_2-terminal serine and threonine residues of proteins [10]. Decrease of rishitin-inducing activity after treatment with Pronase and Proteinase K suggests that the proteinaceous part of CFE might also be important for rishitin induction.

Fractionation of CFE on Sephadex G-200, DEAE-Sephadex A-50 and Concanavalin A-Sepharose 4B.

Figure 4 shows the elution profile of CFE of race 4 on Sephadex G-200. A peak containing nearly all the rishitin-inducing activity ran off shortly after the void volume. Protein and carbohydrate were not separated, so it might be concluded that glycoproteins are involved. The small peaks found later had little rishitin-inducing activity. Rishitin-inducing activity (expressed in µg rishitin per µg glucose equivalents) of the main peak was similar to that of CFE.

CFE of races 4 and 5 was further fractionated on the anion exchanger DEAE-Sephadex A-50. Samples of 50 mg CFE dissolved in 5 ml of 0.02 M potassium phosphate buffer, pH 7, containing 0.02 M NaCl, were applied to a column (1.6 x 30 cm). The unbound fraction was eluted by the same buffer (Fraction I), the bound fraction was partly eluted by a linear 0.02 to 1 M NaCl gradient in the same buffer (Fraction II). Fraction I and II represent together about 60% of the sample applied to the column, (Fraction I about 50%; Fraction II about 10%) which means that about 40% was not eluted by the 0.02 to 1 M NaCl gradient. This fraction could not be removed by increasing the gradient up to 3 M. Fraction I and II were dialyzed against distilled water at 5 °C and lyophilized.

Fifty mg of CFE was also applied to a column (1.6 x 30 cm) containing Concanavalin A covalently linked to Sepharose 4B, which had been equilibrated with 400 ml 0.02 M potassium phosphate buffer, pH 7, containing 10 µM $MnCl_2$ and 10 µM $CaCl_2$ (Con A-buffer). The samples were applied in 4 ml of Con A-buffer. The

unbound fraction was eluted by 40 ml of the same buffer (Fraction III), whereas the bound fraction was only partly eluted by 50 ml of Con A-buffer containing 0.5 M α-D-methylmannoside and α-D-methylglucoside (Fraction IV). Fraction III and IV represent together about 60% of the sample applied to the column (Fraction III ± 56%; fraction IV ± 4%). The fraction still bound to the column after elution by buffer containing both methylglucosides could not be removed by elution with 0.05 M HAc buffer, pH 3, which normally destroys the binding activity of Con A [1]. Fraction III and IV were dialyzed against distilled water and lyophilized. Protein and carbohydrate contents, and rishitin-inducing activities of the four fractions are given in Table 2. Only fraction IV had a higher rishitin-inducing potential than CFE.

Rishitin-inducing activity of elicitors from mycelium extracts (MEE) and cell walls (CWE).

In Fig. 5 the relation between amounts of MEE and CWE of races 4 and 5 of *Cladosporium fulvum* applied to tomato fruit tissue and rishitin production is presented. Maximum rishitin production for MEE of both races of *Cladosporium*

Table 2. Carbohydrate/protein ratio and rishitin-inducing activity of different fractions of elicitors from culture filtrates (CFE) of races 4 and 5, obtained after DEAE-Sephadex and Con A-Sepharose 4B column chromatography.

Fraction	Carbohydrate/protein ratio[a]		Rishitin production as % of CFE control[b]	
	Race 4	Race 5	Race 4	Race 5
CFE[c]	20.1	22.6	100	100
DEAE-Sephadex				
Fraction I	31.0	40.0	56	67
Fraction II	16.5	15.7	14	10
Con A-Sepharose 4B				
Fraction III	74.8	38.1	6	16
Fraction IV	18.0	14.7	309	286

a) Carbohydrate and protein were determined by g.l.c. and amino acid analysis, respectively.

b) 20 µg of glucose equivalents of all fractions was applied to 1 g of tomato fruit tissue; data are from one experiment, representative of a total of three experiments.

c) CFE was prepared as described under Materials and Methods.

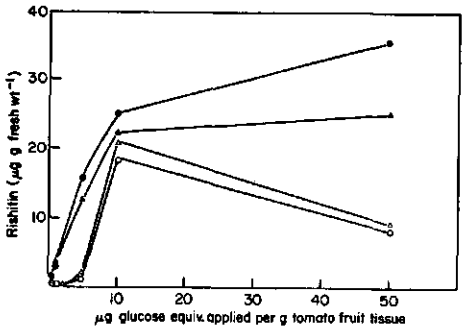

Fig. 5. The relation between amount of mycelium extract (MEE) and cell wall elicitor (CWE) (in µg glucose equivalents per g) applied to tomato fruit tissue and the amount of rishitin (µg g fresh wt^{-1}) produced. ○, MEE race 4; △, MEE race 5; ●, CWE race 4, ▲, CWE race 5.

fulvum was reached by applying 10 to 50 µg glucose equivalents per g fruit tissue. For CWE maximum rishitin production was reached by applying 10 µg glucose equivalents. Fifty per cent of the maximum rishitin production was reached at about 3 and 7 µg for CWE and MEE, respectively. MEE gave an optimum curve with a distinct lag phase, while CWE gave a saturation curve without a distinct lag phase.

Heat and pH stability, and sensitivity to $NaIO_4$ and various enzymes of rishitin-inducing activity of MEE and CWE.

In Table 3 results on heat and pH stability, as well as sensitivity to $NaIO_4$, α-mannosidase, Pronase and Proteinase K on rishitin-inducing activity of MEE and CWE of races 4 and 5 are presented. MEE and CWE behaved similarly to the different treatments as CFE (Table 1). MEE and CWE were even more sensitive to pH 12, α-mannosidase, Pronase and Proteinase K than CFE. These results suggest that in MEE and CWE the same rishitin-inducing principle is present as in CFE.

Specificity of CFE, MEE and CWE of Cladosporium fulvum.

In Table 4 results on the phytoalexin-inducing activity of CFE, MEE and CWE of race 4 of *Cladosporium fulvum* are summarized. Elicitors of all sources of *Cladosporium fulvum* were able to induce pisatin in pea pods and glyceollin in soybean cotyledons. These elicitors were as active as mercuric acetate (30 mg ml^{-1}) and culture filtrate from *Phytophthora megasperma* var. *sojae*, respectively. The *Cladosporium fulvum* elicitors did not induce rishitin in potato tuber discs or medicarpin in Jack beans. Lack of induction of detectable amounts of rishitin and medicarpin was likely not due to an inherent inability of the

Table 3. Heat and pH stability, and sensitivity to $NaIO_4$ and various enzymes of rishitin-inducing activity of elicitors from mycelial extract (MEE) and cell walls (CWE)[a] of races 4 and 5 of *Cladosporium fulvum*.

Treatment[b]	Rishitin production as %[c] of MEE control[d]		Rishitin production as %[c] of CWE control[e]	
	Race 4	Race 5	Race 4	Race 5
Control	100	100	100	100
10 min 100 °C	97	95	88	95
pH 12; 0.01 N NaOH (22 h)	6	8	7	4
pH 2; 0.01 N HCl (22 h)	48	64	65	55
0.02 M $NaIO_4$ (22 h)	0	0	0	0
α-mannosidase, 0.2 mg ml^{-1} (22 h)	23	42	11	6
Pronase, 0.2 mg ml^{-1} (22 h)	<1	1	1	<1
Proteinase K, 0.2 mg ml^{-1} (22 h)	3	5	9	4

a) The MEE and CWE concentrations were 0.1 mg glucose equivalents per ml.
b) For treatments see Materials and Methods.
c) Data are the mean of duplicates from one experiment, representative of a total of three experiments.
d) The absolute values of rishitin production for the MEE controls were 17 and 20 µg rishitin per g for races 4 and 5, respectively.
e) The absolute values of rishitin production for the CWE controls were 22 and 19 µg rishitin per g for races 4 and 5, respectively.
See for other controls also Table 1.

tissues concerned to produce their respective phytoalexins, since *Cladosporium cucumerinum* and *Phytophthora megasperma* f. sp. *medicaginis* did cause phytoalexin accumulation in potato and Jack bean, respectively.

Monosaccharide, amino acid and PO_4 analysis of CFE, MEE and CWE of Cladosporium fulvum.

In Table 5 the data on sugar, protein and PO_4 content of CFE, MEE and CWE of races 4 and 5 of *Cladosporium fulvum* are presented. The carbohydrate/protein ratios of MEE and CWE were about ten times as low as that of CFE. The PO_4 content was highest for MEE which is in accordance with findings of Lloyd [35], who isolated a peptido-phospho-galactomannan complex (PPGM) from *Cladosporium werneckii*.

In Table 6 results on the monosaccharide composition of preparations of CFE, MEE and CWE of races 4 and 5 of *Cladosporium fulvum* are presented. The glucose content of CFE was about two to four times as high as that of MEE and CWE, while the mannose and galactose content of CFE was about ten times as low as that of MEE and CWE. Fraction I containted less and fraction IV about the

Table 4. Phytoalexin-inducing activity of elicitors from culture filtrates (CFE), mycelium extracts (MEE) and cell walls (CWE) of race 4 of *Cladosporium fulvum* in non-hosts of *Cladosporium fulvum*.

Host, tissue tested[a] and phytoalexin isolated	Treatment[b]	Elicitor concentration (µg glucose equivalents per ml)	µg phytoalexin/g[c]
Solanum tuberosum	H_2O	-	1
tuber discs, rishitin	CFE	1	2
	MEE	0.1	1
	CWE	0.1	1
	Cladosporium cucumerinum (10^6 conidia per ml)	-	36
Pisum sativum	H_2O	-	3
pods, pisatin	CFE	1	48
	MEE	0.1	25
	CWE	0.1	27
	mercuric acetate (30 p.p.m.)	-	34
Glycine max	H_2O	-	0
cotyledons, glyceollin	CFE	1	293
	MEE	0.1	336
	CWE	0.1	279
	Phytophthora megasperma var. *sojae* (sterilized culture filtrate)	-	228
Canavalia ensiformis	H_2O	-	0
cotyledons, medicarpin	CFE	1	3
	MEE	0.1	3
	CWE	0.1	2
	Phytophthora megasperma f. sp. *medicaginis* (mycelium)	-	165

a) For preparation of tissues see Materials and Methods.
b) For treatment of tissues with elicitors see Materials and Methods.
c) Data are the mean of duplicates from one experiment representative of two experiments.

Table 5. Sugar, protein and PO_4 content of elicitors, isolated from culture filtrates (CFE), mycelium extracts (MEE) and cell walls (CWE) of races 4 and 5 of *Cladosporium fulvum*.

	weight (%)									
	Sugar[a]		Protein[b]		PO_4		Total		Carbohydrate/ protein ratio	
Origin of elicitor	Race 4	Race 5	Race 4	Race 5	Race 4	Race 5	Race 4	Race 5	Race 4	Race 5
Culture filtrate (CFE)	94.4	95.2	4.7	4.2	0.9	0.6	100	100	20.1	22.7
Mycelium extract (MEE)	45.7	42.6	18.8	19.3	11.5	13.8	76.0	76.2	2.4	2.2
Cell wall (CWE)	64.4	63.3	29.6	28.3	2.3	1.3	96.3	92.9	2.2	2.2

a) Total sugar content was determined by g.l.c. (by summation of the individual monosaccharides).
b) Total protein content was determined by amino acid analysis (by summation of the individual amino acids).

Table 6. Monosaccharide composition of preparations of elicitors isolated from culture filtrates (CFE), mycelium extract (MEE) and cell wall (CWE) of races 4 and 5 of *Cladosporium fulvum*.

Elicitor preparation	Monosaccharide composition (%)							
	Race 4				Race 5			
	rhamnose	mannose	galactose	glucose	rhamnose	mannose	galactose	glucose
Culture filtrate (CFE)	0.89	2.97	2.64	93.70	t[b]	5.22	3.89	90.87
DEAE-sephadex								
Fraction I	0.59	1.42	1.45	96.53	1.81	0.88	0.56	96.75
Fraction II[c]	-	-	-	-	-	-	-	-
Con A-sepharose 4B								
Fraction III	0.37	0	0	99.63	1.22	0	0	98.78
Fraction IV	t[b]	3.02	3.70	93.18	t[b]	4.37	2.02	93.61
Mycelium extract (MEE)	14.76	20.83	24.86	39.55	14.48	26.58	32.65	26.32
Cell walls (CWE)	1.94	22.12	20.18	55.76	1.56	39.49	38.56	21.33

a) Per cent of each monosaccharide in the polysaccharide part of the glycoprotein.
b) t = trace.
c) Monosaccharide composition not determined.

same amount of mannose and galactose as CFE, while in fraction III mannose and galactose were absent.

Amino acid analyzation of CFE, MEE and CWE of races 4 and 5 of *Cladosporium fulvum* (results not presented) showed that all elicitor preparations were especially rich in asparagine/aspartic acid, glutamine/glutamic acid, threonine, serine and proline. There were no striking differences in amino acid composition between the different elicitor preparations.

DISCUSSION

High mol. wt elicitors from culture filtrates (CFE) and especially from mycelial extracts (MEE) and cell wall (CWE) were very potent inducers of rishitin accumulation in tomato fruit tissue. They induced 50 to 10 times as much rishitin as a live conidial suspension of *Cladosporium fulvum*, containing 5 x 10^6 conidia per ml [15]. Besides rishitin, the accumulation of pisatin in pea pods and glyceollin in soybean cotyledons was also induced.

Phytoalexin induction is not the only biological activity of high mol. wt components isolated from *Cladosporium fulvum*. Lazarovits & Higgins [30] and Lazarovits *et al.* [31] isolated glycoproteins from culture filtrates and cell walls of *Cladosporium fulvum* which induced necrosis, callose formation, and electrolyte leakage in tomato leaves. Dow & Callow [19] isolated glycopeptides from culture filtrates of *Cladosporium fulvum* which induced leakage of electrolytes in isolated tomato leaf mesophyll cells. Possibly, in all investigations concerned the same biologically active principle has been tested, however, using different bioassays. In our system we found that tomato fruit tissue which accumulated rishitin also showed some necrosis. It is not known whether necrosis is a cause or consequence of rishitin accumulation.

Lazarovits *et al.* [30, 31] found that glycoproteins isolated from culture filtrates were host-specific with respect to necrosis, callose formation and leakage of electrolytes. However, the phytoalexin-inducing activity of elicitors from *Cladosporium fulvum* was not host-specific, because the accumulation of pisatin in pea pods and glyceollin in soybean cotyledons was also induced. Whereas pisatin and glyceollin synthesis can also be induced by non-specific abiotic elicitors like $HgCl_2$ [39, 49], this chemical did not induce accumulation of rishitin in tomato fruit tissue [15]. Elicitors from *Cladosporium fulvum* did not induce the accumulation of rishitin in potato tuber slices. Lisker & Kuć [34] reported that only heat-killed fungi belonging to the

Oomycetes were able to elicit the accumulation of terpenoids like rishitin in potato tuber slices.

The rishitin-inducing activity of CFE, MEE and CWE was sensitive to reagents which oxidize or cleave polysaccharides ($NaIO_4$ and α-mannosidase) and to enzymes which digest proteins (Pronase and Proteinase K). These results suggest that elicitors from *Cladosporium fulvum* are glycoproteins, which is in agreement with results obtained by Lazarovits *et al.* [31] and Dow & Callow [18]. Lazarovits *et al.* [31] found protein/carbohydrate ratios of about 0.2 for the most purified glycoprotein from culture filtrates of *Cladosporium fulvum*. Dow & Callow [18, 19] found protein/carbohydrate ratios of about 0.3 in fractions with leakage-inducing activity. However, only 40 to 50% of the total weight of these fractions consisted of protein and carbohydrate. Using Lowry's method [37], as modified by Hartree [24], we found protein/carbohydrate ratios of 0.1 to 0.2; when the protein content was based on amino acid analysis data, however, much lower protein/carbohydrate ratios were found. Determination of protein by Lowry's method is possibly influenced by the presence of cladofulvine [44].

The major sugar component of CFE was glucose, while in glycoproteins described by Lazarovits *et al.* [31] and by Dow & Callow [18] it was mannose. The monosugar composition of the glycoproteins secreted by *Cladosporium fulvum* is possibly influenced by the growth medium which was different in all studies, and the method of cultivation (still- or shake-cultures). We also found that the monosugar composition of CFE was different during the exponential and the stationary growth phase (unpublished results). From fractionation studies of CFE on DEAE-Sephadex and Con A-Sepharose 4B the presence of mannose (and galactose) appeared to be more important for the rishitin-inducing activity of the elicitor fractions than glucose. Fraction III consisted almost exclusively of glucose, while its rishitin-inducing activity was low. MEE and CWE contained five to ten times as much mannose and galactose as CFE, while their rishitin-inducing activity was also five to ten times as high as that of CFE. MEE showed some similarities with surface peptido-phospho-galactomannans (PPGM) of the pathogenic yeast, *Cladosporium werneckii* [32, 33, 35, 36], which have antigenic properties, and also with phytotoxic glycopeptides from *Ceratocystis ulmi* [43].

The peptide part of the elicitor fractions was small but seemed to be necessary for rishitin-inducing activity, because Pronase and Proteinase K destroyed the rishitin-inducing activity. It is unlikely that the enzymes Pronase and Proteinase K were contaminated by polysaccharide hydrolyzing enzymes, since neither Pronase nor Proteinase K increased the reducing activity of

elicitor fractions, as assayed by Nelson's test [38], modified by Somogyi [40]. The glycopeptide bond in glycoproteins containing carbohydrate units linked to threonine and serine is split by the process of β-elimination in alkaline conditions [42]. The elicitors from *Cladosporium fulvum* were rich in threonine and serine and were also sensitive to alkali which might indicate the involvement of these amino acid residues in the glycopeptide bonds.

Albersheim & Valent [2] reported that glyceollin-inducing elicitors from *Phytophthora megasperma* var. *sojae* are glucans which are stable at room temperature between pH 2 and 10, and are sensitive to periodate but not to proteases.

Our results, and also those reported by Daniels & Hadwiger [14], Dixon & Fuller [17], and Stekoll & West [41] showed that elicitors of rishitin, pisatin, phaseollin and casbene induction, respectively, were sensitive to Pronase. This indicates that elicitors of phytoalexin induction might be other molecules than glucans.

The structure of elicitors from *Cladosporium fulvum* and the influence of culture conditions are the subject of current research.

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FURTHER CHARACTERIZATION AND CULTIVAR-SPECIFICITY OF GLYCOPROTEIN ELICITORS FROM CULTURE FILTRATES AND CELL WALLS OF *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)

by

P.J.G.M. de Wit and E. Kodde

ABSTRACT

Cladosporium fulvum (syn. *Fulvia fulva*) excretes high-molecular weight glycoproteins into the culture medium which are potent inducers of rishitin accumulation in tomato fruits. The chemical composition of these glycoproteins is dependent of the composition of the growth medium and the age of the culture.

Young cultures produce a glycoprotein that contains nearly only glucose and is not a very active elicitor of rishitin accumulation. At the end of the growth cycle *Cladosporium fulvum* produces a glycoprotein that contains in addition to glucose a high percentage of mannose and galactose. This last glycoprotein is very likely derived from the peptido galactoglucomannan present on the surface of the mycelial wall.

The peptido galactoglucomannan is water soluble and could be extracted from cell walls by water at 121 ^{o}C. The carbohydrate moiety of the peptido galactoglucomannan has a mannose : galactose ratio of 1.21 : 1 and contains traces of glucose. The protein moiety is rich in alanine, asparagine/aspartic acid, glutamine/glutamic acid, proline, serine and threonine. The carbohydrate of the peptido galactoglucomannan is O-glycosidically linked to serine and threonine as was shown by alkaline β-elimination.

There is a strong positive correlation between the mannose and galactose content of the peptido galactoglucomannan and its rishitin and necrosis-inducing activity. The rishitin and necrosis induction by the peptido galactoglucomannan is likely mediated by binding of its terminal mannose and/or galactose residues to the host cell.

The peptido galactoglucomannan appeared to be neither race nor cultivar-specific with respect to the accumulation of phytoalexins in tomato leaves and fruits.

INTRODUCTION

Culture filtrates and cell wall preparations of *Cladosporium fulvum* (syn. *Fulvia fulva*), the causal agent of tomato leaf mould, contain high-molecular glycoproteins which are very potent inducers of rishitin, glyceollin and pisatin accumulation in tomato fruit, soybean cotyledons and pea pods, respectively [9].

Lazarovits *et al.* [27, 28] and Dow & Callow [14, 15] reported that glycoproteins, occurring in culture filtrates and cell walls of *Cladosporium fulvum*, cause callose deposition, necrosis and ion leakage in tomato leaf tissue and electrolyte leakage in tomato mesophyll cells, respectively. Although the biological activity of the glycoproteins concerned was tested in different bioassays, all authors most probably have been working with the same biologically active principle [9, 14, 28]. However, there are some striking differences in the chemical composition of the glycoproteins reported [9, 14, 28], which prompted us to study the influence of (i) the composition of the growth medium and (ii) the age of the cultures on the chemical composition of the glycoprotein elicitor from culture filtrates and cell walls of *Cladosporium fulvum*, because these two factors were different in the reports mentioned.

Cultivar and race-specificity of the purified cell wall elicitor of six races of *Cladosporium fulvum* with respect to induction of rishitin [8, 9] and polyacetylenic phytoalexins [10] in three tomato cultivars with different genes for resistance were also investigated.

MATERIALS AND METHODS

Plants.

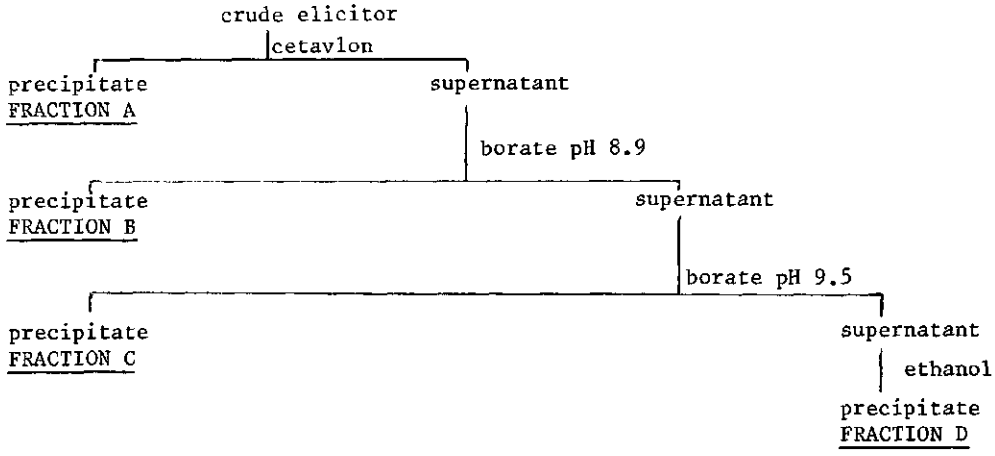
Near-isogenic lines of cv Moneymaker, carrying resistance gene Cf4 or Cf5, and Sonato, carrying resistance gene Cf2Cf4 to *Cladosporium fulvum* were grown in the glasshouse at temperatures between 20 and 28 °C, 60% r.h. and between 10000 and 15000 lx.

Fungi and culture media.

Monospore cultures of *Cladosporium fulvum* race 0, 2, 2.4, 5 and 2.4.5 (kindly supplied by Dr M. Gerlach (Research Institute for Plant Protection, Wageningen), were subcultured on potato dextrose agar (PDA) at 20 °C and stored at 4 °C. To ensure that races maintained their virulence, susceptible tomato plants were inoculated and the pathogen reisolated twice a year. Conidia from two-week-old cultures on PDA were used for initiating liquid shake cultures. Two ml of a conidial suspension (5.10^6 conidia ml^{-1}) were added to 250-ml Erlenmeyers containing 100 ml of medium I as used by Van Dijkman & Kaars Sijpesteijn [40] and by Dow & Callow [14], medium II modified Fries' medium as used by Lazarovits *et al.* [27] or medium III, B5 medium as used by De Wit & Roseboom [9]. The cultures were incubated for 10 or 21 days in the dark at 23 °C on an orbital shaker (180 rev. min^{-1}).

Isolation and fractionation of glycoprotein elicitors.

Culture filtrate elicitor (CFE) was isolated and partly purified as described before [9]. Mycelium was disintegrated by passing it three times through a previously cooled X-Press Cell Disintegrator (LKB-Biotec, Sweden). From this homogenate cell walls and cell wall elicitor (CWE) were prepared by the method described by Ayers *et al.* [3]. Partly purified CFE and CWE were fractionated according to a slight modification of the method described by Lloyd [31]. The protocol of this fractionation is summarized in scheme 1. Two hundred mg of partly



Scheme 1.

purified elicitor was dissolved in 50 ml water where upon 25 ml 7% hexadecyltrimethyl ammoniumbromide (cetavlon) was added. After standing at room temperature overnight, the precipitate was centrifuged and washed with water. The precipitate was dissolved in 10 ml 1 M NaCl by shaking overnight at room temperature. The resulting solution was clarified by centrifugation and three volumes of ethanol were added. The precipitate was isolated by centrifugation and washed with 2% acetic acid in ethanol. The residue was dissolved in 25 ml water, dialyzed, and freeze-dried (Fraction A). To the supernatant (75 ml) 37.5 ml 1% boric acid was added. The solution was stirred and the pH was carefully adjusted to 8.9 by addition of 2 N NaOH. After standing overnight at room temperature the precipitate was centrifuged and washed three times with 0.5% sodium borate, pH 8.9. The residue was dissolved in 10 ml 2% acetic acid, 10 mg sodium acetate was added to the solution and then three volumes of ethanol were added. The resulting precipitate was redissolved in 2% acetic acid and precipitated again. The precipitate was dissolved in 25 ml water, clarified by centrifugation, dialyzed and reisolated by freeze-drying (Fraction B). Redissolution in 2% acetic acid was necessary to remove the last traces of cetavlon and boric acid. The pH of the supernatant was carefully adjusted to 9.5 with 2 N NaOH and the precipitate was isolated as for fraction B (Fraction C). Three volumes of ethanol were added to the supernatant. The resulting precipitate was washed two times with 2% acetic acid in ethanol and then two times with ethanol. The residue was dissolved in 25 ml water, dialyzed and reisolated by freeze-drying (Fraction D).

Bioassays of elicitors and extraction of phytoalexins.

For testing the cultivar-specificity of elicitors tomato fruits and leaves of near-isogenic lines of cv Moneymaker with gene Cf4 (susceptible to race 4, 2.4 and 2.4.5) or Cf5 (susceptible to race 5 and 2.4.5), and of the cultivar Sonato with genes Cf2Cf4 (susceptible to race 2.4 and 2.4.5) were used. Bioassays of elicitors and extraction of rishitin from fruits were carried out as described before [9].

Leaves were treated with aqueous solutions of elicitors by inter-cellular injection with a micro-syringe. Two days after injection the non-sesquiterpenoidal phytoalexins which have been identified as polyacetylenes [10] were extracted and semi-quantitatively assayed as described before [8]. Their purification which required sequential thin-layer chromatographic separations in several solvent systems led to high losses which interfered with their exact quantification.

In some experiments the necrosis-inducing activity of elicitors was determined. An arbitrary rating from 0, ±, +, ++ to +++ was used, indicating no, little, moderate, much and very much necrosis, respectively.

Effect of methylglycosides, polysaccharides and Concanavalin A on the rishitin-inducing activity of purified CWE.

Tomato fruit tissue was preincubated with 50 µl of aqueous solutions of β-methyl-D-glucoside, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-mannoside, galactan, laminarin, mannan, nigeran or Concanavalin A per piece at different concentrations. Four hours after application of the solutions the pieces of tomato fruit tissue were treated with 100 µl of elicitor, the tissue was incubated for two days and rishitin extracted as described elsewhere [9].

Mild acid and alkaline hydrolysis of elicitors.

Portions of elicitors were hydrolyzed at 100 °C in 0.01 N trifluoro acetic acid (TFA) for varying lengths of time. After hydrolysis three volumes of ethanol were added and the component sugars of the resulting precipitate and the remaining supernatant were determined by the method described under Materials and Methods.

Mild alkaline hydrolysis was carried out in 0.05 N NaOH, containing $NaBH_4$ (5 mg ml^{-1}) at 37 °C for 16 hours. After hydrolysis three volumes of ethanol were added and the component sugars of the resulting precipitate and the remaining supernatant were determined by the method described under Materials and Methods.

Alkaline β-elimination of elicitors.

Alkaline β-elimination was carried out according to the method described by Esquerré-Tugayé & Lamport [16]. Elicitor (10 mg) was dissolved in 1 ml N_2H_5OH (24%, Merck) and incubated at 110 °C in a sealed tube. After 18 hours the tube was opened, and the solution dried under a stream of nitrogen. The residue was hydrolyzed in 6 N HCl at 110 °C for 24 hours and the amino acids determined as described under Materials and Methods. The amino acid compositions of elicitors with and without prior treatment with N_2H_5OH were compared to assess the amount of glycosylated serine and threonine.

Methylation analysis of elicitors.

Methylation analysis was carried out by the method of Hakomori [20] as described by Sanford & Conrad [39]. Elicitor (10 mg) was dissolved in 2 ml dimethylsulfoxide (DMSO) by heating at 40 °C in a 25-ml flask fitted with a serum stopper. The flask was then flushed with nitrogen using a hypodermic needle and 1.5 ml of a solution of freshly prepared dimethylsulfinyl sodium in DMSO was added drop-wise with a syringe. After stirring for four hours, the solution was cooled and 0.5 ml methyliodide was added drop-wise with a syringe. Stirring was continued overnight. The excess of methyliodide was removed by evaporation under reduced pressure at 40 °C; the remaining solution was dialyzed overnight, and lyophilized. Five mg of the lyophilized materials was hydrolyzed in 3 ml 90% formic acid at 100 °C for four hours and then in 3 ml 0.25 M H_2SO_4 at 100 °C for 16 hours. The solution was neutralized with $Ba(OH)_2$, and the precipitate centrifuged. The supernatant was neutralized, 20 mg $NaBH_4$ added, and the solution incubated overnight at room temperature. The excess of $NaBH_4$ was decomposed by addition of acetic acid. A spatula of Dowex-50W-X8(H^+) was added to the solution and after stirring for one hour the cation exchange resin was removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure at 40 °C and washed twice with methanol. Four ml of acetic acid/pyridine (1 : 1, v/v) was added and the solution incubated for 20 min at 100 °C. The reagent was evaporated to dryness at 40 °C and the residue dissolved in chloroform.

The partial methylated alditol acetates were analyzed by gas liquid chromatography (g.l.c.) on a 3% OV 225 chromosorb WHP 100-120 mesh; they were identified by their relative retention [5] and by combined gas chromatographic and mass spectrometric analysis (g.c.-m.s.).

Determination of phosphorus, carbohydrate and protein.

Phosphorus was determined by the method of Chen *et al.* [7]. Hexoses were assayed by the anthrone method [11] with D-glucose as a standard. Protein was determined by the method of Lowry *et al.* [33] as modified by Hartree [21] using bovine serum albumin (BSA) as a standard.

Identification of sugars and amino acids.

The component sugars of elicitors were determined by the method of Jones & Albersheim [23] as their alditol acetates. G.l.c. of alditol acetates was carried out on a 250 cm x 2 mm i.d. glass column packed with 0.6% OV 275 and 0.8% XF 1150 on diatomite CQ 100 to 120 mesh (Chrompack). Nitrogen was used as the carrier gas at a flow rate of 40 ml min^{-1} and detection was by flame

ionization. Operating temperatures were as follows: injection port 250 °C, detector 250 °C and column 200 °C. Inositol was used as an internal standard.

Amino acids in the protein part of the glycoprotein elicitors were determined by g.l.c. as the N-heptafluorobutyrylisopropyl esters according to the method of Kaiser *et al.* [24] after hydrolysis in 6 N HCl at 110 °C for 24 hours.

Column chromatography.

Sephadex G-200, Sepharose 6B and Concanavalin A covalently linked to Sepharose 4B were obtained from Pharmacia. The equilibration buffers, and column dimensions are described in the text.

RESULTS

The influence of growth medium and age of cultures on the chemical composition of culture filtrate (CFE) and cell wall elicitors (CWE).

Table 1 presents the results on the influence of the growth medium on the chemical composition of CFE of ten-day-old cultures of six races of *Cladosporium fulvum*. From this table it is clear that CFE of all races grown on medium I contained, next to glucose, a great deal of mannose and galactose, while, with the exception of race 2, CFE of all races grown on medium II and III contained mainly glucose and only traces of mannose and galactose. Elicitors of all races grown on medium I and of race 2 grown on medium II and III showed the highest rishitin-inducing activity. Thus rishitin induction appears positively correlated with a high mannose and galactose content of CFE.

In Table 2 the results of 21-day-old cultures are presented. This table shows that the CFE of all races grown on all three media contains next to glucose also mannose and galactose. The mannose content of CFE of race 2 was high compared with that of CFE of other races. The positive correlation between mannose and galactose content of CFE and its rishitin-inducing activity was not as strong as in Table 1.

Table 3 shows the results on the influence of the growth medium on the chemical composition of CWE of ten-day-old cultures of six races of *Cladosporium fulvum*. CWE of all races grown on the three different media contained, in addition to glucose, much mannose and galactose, except race 0 and 2.4.5, grown on medium I and II. This table shows again a positive correlation between

Table 1. The influence of the growth medium (I, II and III) on the chemical composition and rishitin-inducing activity of CFE of 10-day-old cultures of six races of *Cladosporium fulvum*.

growth medium	race	carbohydrate/protein ratio[a]	monosaccharide ratio (%)			rishitin[b] µg g^{-1}
			mannose	galactose	glucose	
I	0	21.2	4	4	92	8.5
	2	5.0	61	22	17	23.3
	4	4.8	23	18	59	13.4
	2.4	2.6	27	22	51	21.7
	5	1.7	33	19	48	22.9
	2.4.5	9.5	14	9	77	11.6
II	0	65.7	t[c]	t	99	1.2
	2	1.6	69	16	15	19.8
	4	10.8	3	1	96	3.4
	2.4	24.6	t	t	99	3.0
	5	18.6	t	t	99	5.2
	2.4.5	61.5	t	t	99	2.2
III	0	18.2	t	t	99	7.6
	2	1.8	33	24	43	15.4
	4	18.2	t	t	99	4.0
	2.4	4.5	8	6	86	6.4
	5	5.8	4	2	94	3.6
	2.4.5	23.4	2	1	97	2.7

a) Total sugar content was determined by g.l.c. (summation of the individual monosaccharides); Total protein content was determined by the method of Lowry *et al.* [33] as modified by Hartree [21] with BSA as a standard; phosphate was present in trace amounts.

b) Rishitin production was determined with tomato fruits of the cultivar Sonato (Cf2Cf4); the elicitor concentration was 1.0 mg ml^{-1}; data are the mean of two replicates. The correlation coefficients between the mannose, galactose and glucose content of CFE and its rishitin-inducing activity were 0.87 (0.1% significance), 0.93 (0.1% significance) and -0.92 (0.1% significance), respectively.

c) t : trace, less than 0.5%.

mannose and galactose content of CWE and its rishitin-inducing activity. CWE prepared from races grown for three weeks in liquid shake cultures was not significantly different from CWE of ten-day-old cultures with respect to its chemical composition and its rishitin-inducing activity (results not presented). Usually CWE was more active than CFE. A lower concentration of CWE (0.25 mg ml^{-1}) compared with CFE (1 mg.ml^{-1}) was needed to induce the same amount of rishitin.

Table 2. The influence of the growth medium (I, II and III) on the chemical composition and rishitin-inducing activity of CFE of 21-day-old cultures of six races of *Cladosporium fulvum*.

growth medium	race	carbohydrate/protein ratio[a]	monosaccharide ratio (%)			rishitin[b]
			mannose	galactose	glucose	µg g^{-1}
I	0	19.2	10	8	82	10.4
	2	9.3	51	26	23	15.3
	4	14.8	33	19	48	17.2
	2.4	9.2	9	10	81	6.9
	5	6.4	18	11	71	13.2
	2.4.5	7.8	14	16	70	5.7
II	0	7.8	40	16	44	7.7
	2	5.1	71	13	16	21.3
	4	-[c]	-	-	-	-
	2.4	5.8	34	18	48	8.7
	5	7.1	38	12	50	8.8
	2.4.5	18.9	9	4	87	8.3
III	0	14.1	6	4	90	4.2
	2	3.7	43	30	27	11.2
	4	21.1	17	16	67	8.0
	2.4	9.3	42	29	29	13.7
	5	5.4	16	12	72	3.9
	2.4.5	20.2	4	3	93	3.3

a) Details are as in Table 1; phosphate was present in trace amounts up to 2.2% (weight).

b) Rishitin production was determined with tomato fruits of the cultivar Sonato (Cf2Cf4); the elicitor concentration was 1.0 mg ml^{-1}; data are the mean of two replicates. The correlation coefficents between the mannose, galactose and glucose content of CFE and its rishitin-inducing activity were 0.77 (0.1% significance), 0.47 (3% significance) and -0.74 (0.1% significance), respectively.

c) Sample lost.

Fractionation of CFE and CWE.

CFE and CWE were fractionated as summarized in Scheme 1. Table 4 shows the results on the fractionation with CFE of race 4 grown in the three different media. Yield as well as monosaccharide compositions of fraction A, B, C and D were different from each other. The rishitin and necrosis-inducing activity of the different CFE-fractions were positively correlated with their mannose and galactose content. Table 5 shows the results on the fractionation of CWE. From this table it is clear that fraction B of CWE gave the highest yield on

Table 3. The influence of the growth medium (I, II and III) on the chemical composition and the rishitin-inducing activity of CWE of 10-day-old cultures of six races of *Cladosporium fulvum*.

growth medium	race	carbohydrate/protein ratio[a]	monosaccharide ratio (%)			rishitin[b] µg g^{-1}
			mannose	galactose	glucose	
I	0	5.2	18	12	70	1.4
	2	2.6	50	25	25	9.6
	4	2.3	39	33	28	17.9
	2.4	3.2	31	24	45	7.5
	5	1.6	41	30	29	16.8
	2.4.5	3.7	19	16	65	2.4
II	0	5.0	10	5	85	8.2
	2	1.9	50	30	20	30.1
	4	6.9	40	31	29	17.5
	2.4	-[c]	-	-	-	-
	5	3.3	45	28	27	15.5
	2.4.5	4.2	16	12	72	6.2
III	0	2.2	34	24	42	20.5
	2	2.6	49	15	36	26.6
	4	4.5	35	32	33	17.1
	2.4	2.0	56	35	9	-
	5	2.8	42	25	33	27.0
	2.4.5	2.4	37	26	37	31.7

a) Details are as in Table 1; phosphate was present in trace amounts up to 3% (weight).

b) Rishitin production was determined with tomato fruits of the cultivar Sonato (Cf2Cf4); the elicitor concentration was 0.25 mg ml^{-1}. Data are the mean of two replicates. The correlation coefficients between mannose, galactose and glucose content of CWE and its rishitin-inducing activity were 0.69 (0.1% significance), 0.51 (2% significance) and -0.66 (0.3% significance), respectively.

c) Sample lost.

all three media. Fraction B had a fairly constant mannose : galactose : glucose ratio and a very high rishitin and necrosis-inducing activity.

Table 6 shows the chemical composition as well as the rishitin and necrosis-inducing activity of fraction B of CWE of six races of *Cladosporium fulvum* grown on medium III. The mannose : galactose ratio was always about 1.2 : 1, while the glucose content was slightly variable. All B-fractions had very high rishitin and necrosis-inducing activity.

Table 4. Monosaccharide ratio (%) and rishitin and necrosis-inducing activity of CFE and fractions A, B, C and D of CFE of 21-day-old cultures of race 4 of *Cladosporium fulvum*.

growth medium	elicitor[a]	yield (%)	monosaccharide ratio (%)			rishitin[b] µg g^{-1}	necrosis[c]
			mannose	galactose	glucose		
I	CFE	100	33	19	48	17.2	++
	fraction A	14	15	4	81	8.9	+
	fraction B	62	44	25	31	22.6	++
	fraction C	24	6	2	92	4.2	±
	fraction D	-[d]	-	-	-	-	-
II[e]	CFE	100	3	1	96	3.4	±
	fraction A	92	4	1	95	4.8	±
	fraction B	8	3	1	96	3.7	±
	fraction C	-	-	-	-	-	-
	fraction D	-	-	-	-	-	-
III	CFE	100	17	16	67	8.0	+
	fraction A	24	35	39	26	9.3	+
	fraction B	15	49	40	11	26.1	++
	fraction C	1	1	1	98	1.3	-
	fraction D	60	1	1	98	1.0	-

a) The elicitor concentration was 1.0 mg ml^{-1}.

b) Rishitin production was determined in fruits of cultivar Sonato (Cf2Cf4); data are the mean of two replicates. The correlation coefficients between the mannose, galactose and glucose content of all fractions of CFE and their rishitin-inducing activity were 0.95 (0.1% significance), 0.79 (0.1% significance) and -0.89 (0.1% significance), respectively.

c) ++ much necrosis, + moderate necrosis, ± little necrosis, - no necrosis.

d) No yield.

e) 10-Day-old culture of race 4.

Table 5. Monosaccharide ratio (%) and rishitin and necrosis-inducing activity of CWE and fractions A, B, C and D of CWE of 10-day-old cultures of race 4 of *Cladosporium fulvum*.

growth medium	elicitor[a]	yield (%)	monosaccharide ratio (%)			rishitin[b] µg g^{-1}	necrosis[c]
			mannose	galactose	glucose		
I	CWE	100	39	33	28	17.9	++
	fraction A	19	27	25	48	4.4	+
	fraction B	56	47	43	10	23.9	+++
	fraction C	25	26	22	52	7.5	+
	fraction D	-[d]	-	-	-	-	-
II	CWE	100	40	31	29	17.5	++
	fraction A	9	14	11	75	4.0	+
	fraction B	73	48	44	8	26.3	+++
	fraction C	18	3	2	95	4.6	±
	fraction D	-	-	-	-	-	-
III	CWE	100	35	32	33	17.1	++
	fraction A	25	9	11	80	2.3	+
	fraction B	70	46	42	12	25.0	+++
	fraction C	5	2	4	94	1.7	±
	fraction D	-	-	-	-	-	-

a) The elicitor concentration was 0.25 mg ml^{-1}.

b) Rishitin production was determined in tomato fruits of the cultivar Sonato (Cf2Cf4); data are the mean of two replicates. The correlation coefficients between the mannose, galactose and glucose content of all fractions of CWE and their rishitin-inducing activity were 0.93 (0.1% significance), 0.93 (0.1% significance) and -0.93 (0.1% significance), respectively.

c) +++ very much necrosis, ++ much necrosis, + moderate necrosis, ± little necrosis, - no necrosis.

d) No yield.

Table 6. Chemical composition, rishitin and necrosis-inducing activity of fraction B from CWE of race 0, 2, 4, 2.4, 5 and 2.4.5 of *Cladosporium fulvum* grown on medium III.

race	carbohydrate/protein ratio[a]	monosaccharide ratio (%)			rishitin[b] µg g^{-1}	necrosis[c]
		mannose	galactose	glucose		
0	5.9	49	38	13	19.4	+++
2	5.0	54	38	8	20.1	+++
4	5.5	46	42	12	25.0	+++
2.4	5.8	52	41	7	20.6	+++
5	5.8	49	40	11	33.7	+++
2.4.5	6.9	49	38	13	18.3	+++

a) Details are as in Table 1; phosphate was present in trace amounts (less than 0.5%, weight).

b) Rishitin production was determined with tomato fruits of the cultivar Sonato (Cf2Cf4); the elicitor concentration was 0.25 mg ml^{-1}; data are the mean of two replicates.

c) +++ very much necrosis.

Cultivar-specificity of fraction B of CWE.

Table 7 shows the results of the production of rishitin in tomato fruit tissue of cultivars carrying resistance genes Cf4, Cf5 or Cf2Cf4 to *Cladosporium fulvum* after treatment with fraction B of CWE of six races of this fungus. The amount of rishitin, induced by fraction B of different races, was neither cultivar nor race-specific. Also the polyacetylenic phytoalexins falcarinol and falcarindiol [10] were induced in tomato fruit as well as in tomato leaf tissue after treatmen with the same fractions. Here again, there was no cultivar-specificity (results not presented). The leaf bioassay, though semi-quantitative, was sufficiently reliable to draw this conclusion. Fraction B of CWE of all races of *Cladosporium fulvum* induced necrosis in all cultivars, irrespective whether the interaction was compatible or not.

Further chemical characterization of fraction B of CWE of race 4 of Cladosporium fulvum.

Fraction B of CWE of race 4 of *Cladosporium fulvum* (10 mg) was applied to a column (1.6 x 30 cm) containing Concanavalin A, covalently linked to Sepharose

Table 7. Rishitin-inducing activity of fraction B[a] of CWE from six races of *Cladosporium fulvum* in tomato fruits carrying resistance genes Cf4, Cf5 and Cf2Cf4.

race	Cf4		Cf5		Cf2Cf4	
	rishitin µg g^{-1} [b]	cultivar-race interaction[c]	rishitin µg g^{-1}	cultivar-race interaction	rishitin µg g^{-1}	cultivar-race interaction
0	11.7	I	13.2	I	15.9	I
2	17.3	I	16.9	I	14.5	I
4	15.0	C	16.2	I	19.2	I
2.4	10.3	C	14.6	I	16.3	C
5	18.1	I	20.3	C	17.8	I
2.4.5	17.1	C	18.2	C	19.6	C

a) Elicitor concentration was 0.25 mg ml^{-1}.

b) Data are the mean of two replicates.

c) I: incompatible, C: compatible.

4B, which had been equilibrated with 400 ml 0.02 M potassium phosphate buffer, pH 7, containing 10 µM $MnCl_2$ and 10 µM $CaCl_2$ (Con A-buffer). Fraction B of CWE was bound almost completely to the column and could completely be eluted by 0.5 M α-D-methylmannoside in Con A-buffer.

Fraction B of CWE applied to Sepharose 6B and eluted with 0.02 M phosphate buffer, pH 7, showed two peaks (Fig. 1). The second peak (indicated as III in Fig. 1) applied to Sephadex G-200 showed only one main peak (results not presented). Monosaccharide compositions of the peaks eluted from the Sepharose 6B and

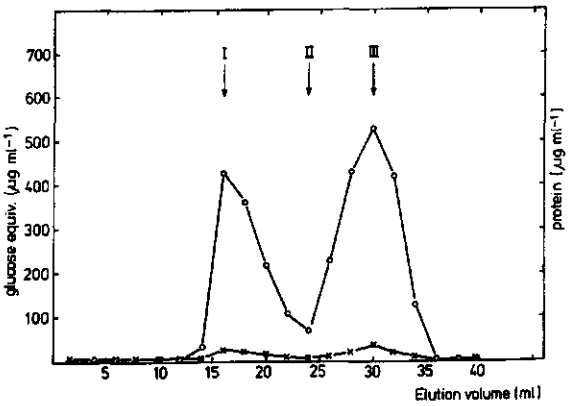


Fig. 1. Chromatography of fraction B of CWE of race 4 of *Cladosporium fulvum* on Sepharose 6B. A sample dissolved in 1 ml 0.02 M potassium phosphate buffer, pH 7, was applied to a column (1.6 x 30 cm; total volume 40 ml) and fractions of 2 ml were collected. The arrows indicate fractions (I, II and III), the monosaccharide composition of which is given in Table 8 (O glucose equiv.; × protein).

Table 8. Monosaccharide ratio (%) of fractions eluted from Sepharose 6B and Sephadex G-200 columns.

fraction	monosaccharide ratio (%)		
	mannose	galactose	glucose
Sepharose 6B[a)]			
I	49.1	41.7	9.2
II	52.6	43.5	3.9
III	54.0	44.6	1.4
Sephadex G-200 I[b)]	54.1	44.5	1.4

a) Fractions correspond with I, II and III in Fig. 1.

b) Fraction corresponds with top of the peak eluted from the column.

Sephadex G-200 column are shown in Table 8. From this table it is obvious that the mannose : galactose ratio of fraction B of CWE is constant in all fractions eluted from both columns, but that the glucose content is significantly different in fraction I, II and III of the Sepharose 6B column. When fraction III of the Sepharose 6B column was applied to a G-200 column, then the mannose : galactose : glucose ratio did no longer differ significantly, in the main peak, indicating that fraction III of the Sepharose 6B column was sufficiently pure. This fraction will be called pure CWE.

Methylation analysis of the carbohydrate moiety of pure CWE showed tetramethyl-derivatives of mannose, but mainly of galactose, indicating that these two sugar residues are located at non-reducing termini. All terminal galactose residues were in the D-furanosyl form (f). The main chains consisted of 1 → 2 linked mannose and 1 → 6 linked galactose (f). The chains were heavily branched, the mannose chain mainly at the 6-position and the galactose chain at the 5-position.

Hydrolysis of pure CWE under mild acid conditions released nearly 100% of the galactose residues after one hour, indicating again that the galactose residues are in the furanose form (Fig. 2). Prolonged hydrolysis led to release of some glucose and mannose.

Mild alkaline hydrolysis of pure CWE released oligomers containing mainly mannose and some galactose and glucose residues. The released oligomers accounted

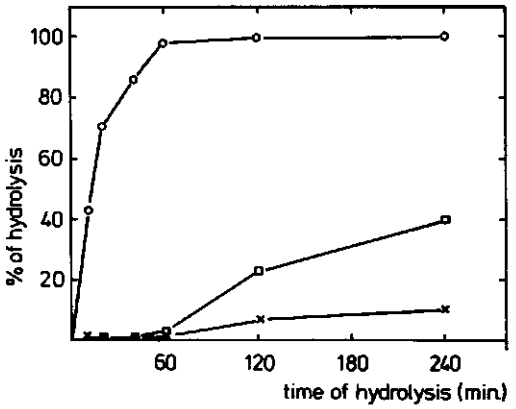


Fig. 2. Partial acid hydrolysis of fraction III eluted from the Sepharose 6B column (Fig. 1) in 0.01 N trifluoroacetic acid at 100 °C for varying lengths of time. Monosaccharides (%) present in the supernatant of the partial hydrolyzed fraction III, after addition of three volumes ethanol, were determined, without prior hydrolysis of the supernatant in 2N TFA (○ galactose; □ glucose; × mannose).

for 5 - 7% of the total carbohydrate of pure CWE. These oligomers are very likely involved in the O-glycosidic linkage between carbohydrate and protein.

The amino acid composition of pure CWE is shown in Table 9. The glycoprotein is rich in alanine, threonine, serine, proline, asparagine/aspartic acid, glutamine/glutamic acid. Treatment with N_2H_5OH caused β-elimination and a concomitant decrease in threonine and serine, indicating that threonine and serine are involved in the O-glycosidic linkage between carbohydrate and protein.

Effect of methylglycosides, polysaccharides, and Concanavalin A on the rishitin-inducing activity of pure CWE.

In this experiment we tried to find out whether methylglycosides, polysaccharides or Concanavalin A could compete for the elicitor-binding sites of tomato fruit tissue. Ayers *et al.* [4] and Marcan *et al.* [34] found inhibitory effects of such compounds on the elicitor activity. In Table 10 the results of this experiment are shown. Only at high concentrations β-D-methylgalactoside, α-D-methylmannoside and galactan significantly inhibited the rishitin-inducing activity of purified CWE, which suggests that the rishitin induction by the peptido galactoglucomannan is mediated by binding of its terminal mannose or/and galactose residues to the host cell. Concanavalin A, on the other hand, inhibited the rishitin-inducing activity at all concentrations tested.

Effect of mild acid and alkaline hydrolysis of pure CWE on its rishitin-inducing activity.

Mild acid and alkaline hydrolysis inhibited the rishitin-inducing activity of pure CWE for about 40% and 97%, respectively. Removing the short mainly

Table 9. Amino acid composition (%) of purified CWE[a] of race 4 of *Cladosporium fulvum* with and without treatment with N_2H_5OH.

amino acid	$-N_2H_5OH$	$+N_2H_5OH$[b]
ala	12.7	16.4
gly	7.8	9.1
val	8.8	9.7
thr	12.7	5.3
ser	13.7	6.4
leu	2.9	3.8
pro	12.7	15.6
asp	11.8	12.4
glu	14.7	16.9
phe	2.1	4.4

a) Fraction III corresponding with III in Fig. 1; the protein content was 4% as determined by amino acid analysis (summation of individual amino acids); the amino acids not mentioned occurred in trace amounts (less than 0.5%, weight).

b) 25% N_2H_5OH for 18 hours at 110 °C.

mannose-containing oligomers of pure CWE (alkaline hydrolysis) seems to have a more dramatic effect on the rishitin-inducing activity of the remaining polymer than removing its galactofuranosyl residues (acid hydrolysis). These results again suggest that the rishitin induction by the peptido galactoglucomannan is mediated by binding of terminal mannose or/and galactose residues to the host cell.

DISCUSSION

Many fungi are known to produce extracellular glycoproteins in liquid culture media [17]. The growth conditions sometimes influence the monosaccharide composition of these glycoproteins. For example, the percentage of galactose of the 5-O-β-D-galactofuranosyl-containing extracellular glycopeptide of *Penicillium charlesii* decreased from 65% in six-day-old cultures to 12 - 15% in 20-day-old cultures [17]. This decrease in percentage of galactose resulted from partial hydrolysis due to an extracellular exo-β-D-galactofuranosidase that accumulated in the medium between the sixth and the fifteenth day. In

Table 10. Effect of methylglycosides, polysaccharides and Concanavalin A on the rishitin-inducing activity of purified CWE[a].

treatment[b]	concentration	inhibition (%)[c]
α-D-methylgalactopyranoside	up to 1 M	0
β-D-methylgalactopyranoside	10^{-3} M	0
	10^{-1} M	28
	1 M	38
β-D-methylglucopyranoside	up to 1 M	0
α-D-methylmannopyranoside	10^{-1} M	0
	1 M	36
galactan	1 mg ml^{-1}	0
	10 mg ml^{-1}	15
	50 mg ml^{-1}	44
laminarin	up to 10 mg ml^{-1}	0
mannan	up to 10 mg ml^{-1}	0
nigeran	up to 0.5 mg ml^{-1}	0
Concanavalin A	0 mg ml^{-1}	0
	0.1 mg ml^{-1}	33
	0.5 mg ml^{-1}	51
	2.5 mg ml^{-1}	71
	5.0 mg ml^{-1}	86

a) Fraction III, corresponding with fraction III in Fig. 1; elicitor concentration was 0.25 mg ml^{-1}.

b) The compounds used in the different treatments did not induce rishitin.

c) The rishitin accumulation was determined with tomato fruits of the cultivar Sonato (Cf2Cf4); the amount of rishitin induced in tomato fruit tissue treated with 50 µl water and then 100 µl elicitor (0.25 mg ml^{-1}) per piece was used as control (0% inhibition); data are the mean of two replicates.

addition, stationary cultures of *Penicillium charlesii* produced extracellular glycopeptides which contained mannosyl, glucosyl and galactofuranosyl residues while shake cultures contained glycopeptides without glucosyl residues [18].

In this paper we have compared the chemical composition of culture filtrate elicitor (CFE) obtained from culture filtrates of *Cladosporium fulvum*, grown in the same media as described by De Wit & Roseboom [9], Dow & Callow [14] and Lazarovits *et al.* [28]. Growth conditions, indeed, strongly influenced

the chemical composition of CFE. The excreted glycoproteins may be either intrinsically different in the three media or differently degraded by extracellular polysaccharide degrading enzymes occurring in culture filtrates. Thus, comparison of glycoproteins excreted into the culture medium by *Cladosporium fulvum* is only justified if the fungus is cultured under strictly controlled conditions.

The chemical composition of cell wall elicitor (CWE), on the other hand, was independent of the composition of the growth medium and the age of the culture. Fractionation of CWE by hexadecyltrimethyl ammonium bromide in borate buffer yielded a peptido heteroglycan with a mannose : galactose ratio of 1.21 : 1 and traces of glucose. Purified CWE is a peptido galactoglucomannan and a very active elicitor of rishitin accumulation. Purified CWE has also a high necrosis-inducing activity, suggesting that it also behaves as a toxin [27, 28]. It is not known whether necrosis is a cause or consequence of rishitin accumulation. In all elicitor preparations, but especially in the purified ones, there was a significant positive correlation between the mannose and galactose content and the rishitin and necrosis-inducing activity.

The peptido galactoglucomannan of *Cladosporium fulvum* shares many structural characteristics with peptido galactomannans from *Cladosporium werneckii* [29, 30, 31, 32], the proteo galactoglucomannan from *Piricularia oryzae* [35, 36] and the 5-O-β-D-galactofuranosyl-containing extracellular glycopeptide from *Penicillium charlesii* [18, 38]. Especially the non-reducing terminal galactofuranosyl residues and the 1 → 2 mannose linkage in the main chain are typical common characteristics.

Peptido heteroglycans are macromolecules present on the cell surface. They are of special interest, because of their involvement in numerous cell functions, such as wall synthesis and cell-cell interactions, and their role in the immunological properties of the cells [29, 32, 35, 36]. The immuno-determinant groups are either terminal mannose, galactose or glucose residues, or glycosyl chains [30, 36].

In order to determine whether rishitin induction was mediated by binding of terminal galactose or mannose residues of the peptido galactoglucomannan of *Cladosporium fulvum* to the host cell, the effect of methylglycosides, oligosaccharides and Concanavalin A on the rishitin induction was studied. Only high concentrations of α-D-methylmannoside, β-D-methylgalactoside and galactan significantly inhibited the rishitin-inducing activity of the peptido galactoglucomannan. The inhibition was likely specific, because high concentrations of other methyl glycosides and polysaccharides did not cause inhibition. With

Concanavalin A we could also significantly inhibit the rishitin-inducing activity, but the higher concentrations of Concanavalin A caused some precipitation of the peptido galactoglucomannan which interfered with the measurement of inhibition. Mild acid, but especially mild alkaline hydrolysis of the peptido galactoglucomannan, removing its galactofuranosyl residues and its mannose-containing oligomer side chains, respectively, gave also a significant inhibition of rishitin-inducing activity. This result again suggests that binding of mannose or/and galactose residues of the peptido galactoglucomannan to the host cell, is a prerequisite for rishitin induction.

The glycoprotein elicitors appearing in the medium at the end of the growth cycle [14, 28] are likely derived from the peptido galactoglucomannan present on the surface of the cell wall. However, CFE excreted into the medium by young cultures, containing nearly only glucose, is significantly different from the peptido galactoglucomannan and probably belongs to another group of macromolecules. CFE of young cultures is neither an active elicitor of rishitin accumulation. This suggests that induction of phytoalexin accumulation *in vivo* is caused by the peptido galactoglucomannan of the cell wall. Direct contact between plant cell and fungal cell seems to be a prerequisite for elicitation of phytoalexins.

The peptido galactoglucomannan appeared to be neither race nor cultivar-specific with respect to the accumulation of phytoalexins and induction of necrosis. Lazarovits *et al.* [27, 28] and Dow & Callow [14, 15] also found their glycoprotein toxins and glycopeptides to be neither race nor cultivar-specific with respect to electrolyte loss from treated tomato tissue. These findings are in contrast with those of Van Dijkman & Kaars Sijpesteijn [40] who found high-molecular weight toxins in culture filtrates of *Cladosporium fulvum* which showed cultivar-specificity with respect to leakage of preabsorbed ^{32}P from treated tomato leaf discs. In some other host-pathogen interactions it has also been found that elicitors of phytoalexin accumulation are neither race nor cultivar-specific [1, 2, 4]. It might be possible that elicitors are just inducers of a general defence reaction.

Thus, we still have to look for the "specificity factor" in the system *Cladosporium fulvum*-tomato which accounts for the race-specific resistance. With respect to the systems *Phytophthora megasperma* var. *sojae*-soybean and *Pseudomonas glycinea*-soybean, respectively, Keen and coworkers [6, 25, 26] claim the presence of race-specific elicitors of phytoalexin accumulation, while Wade & Albersheim [41] postulate the presence of glycopeptides produced by avirulent races of the same pathogen which could protect soybean plants from

attack by virulent ones and which themselves have only weak elicitor activity. A common feature of the models of Keen and coworkers [6, 25, 26] and Wade & Albersheim [41] is that specificity is associated with the incompatible interaction. However, this may not always be the case. Doke *et al.* [12], Garas *et al.* [19] and Doke & Tomiyama [13] suggest that in the system *Phytophthora infestans* - potato specificity is associated with the compatible interaction. Virulent races of *Phytophthora infestans* contain β-1,3 and β-1,6 linked low-molecular weight glucans which could inhibit the hypersensitive reaction and the accumulation of terpenoid phytoalexins. Oku *et al.* [37] and Heath [22] found comparable results with *Mycosphaerella pinodes* and rust fungi, respectively.

Current research in the system *Cladosporium fulvum*-tomato is concentrated on the occurrence of substances in culture filtrates of the fungus and intercellular fluids of infected tomato leaf tissue which specifically inhibit or suppress the phytoalexin accumulation induced by elicitors.

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GENERAL DISCUSSION

The aim of this study was to obtain a better understanding of the physiological nature of cultivar-specific resistance of tomato to *Cladosporium fulvum*.

Fluorescence microscopy showed that, under optimal conditions for infection, callose deposition was associated with incompatible interactions (first paper [14]. From additional light and scanning-electron microscopic time course studies, it was impossible to judge whether this host response resulted in, or was caused by incompatibility. Callose deposition is a cell response to many stimuli [2, 11, 34] and it is unlikely that this response is specifically induced by avirulent races of *Cladosporium fulvum*. The function of callose deposition in plants is not known yet. It is suggested that callose deposition may play a role in localization of viruses within lesions occurring in hypersensitively reacting hosts [36, 37]. Such a diffusion barrier-function of callose in the combination *Cladosporium fulvum* - tomato would be unlikely, since *Cladosporium fulvum* grows intercellularly without formation of haustoria, while callose is deposited intracellularly. It could be possible that callose prevents leakage of nutrious substances from the host cells and thus indirectly inhibits fungal growth. Callose deposition in incompatible interactions of *Cladosporium fulvum* and tomato was also reported by Lazarovits & Higgins [31, 32].

A protein of host origin, synthesized *de novo* appeared more rapidly in incompatible than in compatible interactions (second paper [15]). Although its appearance was strongly associated with the hypersensitive response, which is very localized, the protein itself was systemically induced to some extent in control leaflets of an inoculated compound leaf. This suggest that the inducer of the protein is systemic. It is unlikely that the protein itself would be translocated [9]. The protein is appearantly not an enzyme involved in phytoalexin synthesis, since in leaves, which contained the systemically induced protein, no phytoalexins could be detected (unpublished results). In contrast to the systemically induced protein in virus-infected plants, which are thought to be associated with acquired systemic resistance [43, 44], mycelial growth of virulent races of *Cladosporium fulvum* was not inhibited in tomato leaves containing the systemically induced protein. Systemically induced resistance as in cucumber, watermelon and muskmelon against *Colletotrichum lagenarium* [10, 30] has not yet been observed in tomato plants

against *Cladosporium fulvum*. The fact that the protein is already present in healthy tomato plants, even though in very low amounts, suggests that its accumulation should not be regarded as a specific response of genes for resistance directed to *Cladosporium fulvum*. The appearance of the protein is more likely a consequence rather than a cause of the hypersensitive response.

In tomato leaves two phytoalexins accumulated more rapidly in incompatible than in compatible interactions, while in fruits no differential accumulation of these phytoalexins, and the sesquiterpene rishitin occurred (third paper [16]). In leaves fungal growth inhibition coincided with accumulation of phytoalexins.

The two above-mentioned phytoalexins were identified as being the polyacetylenes falcarindiol and probably falcarinol (fourth paper [17]). Although the level to which the polyacetylenes accumulated *in vivo* nearly reached the ED_{50}-values of hyphal and mycelial growth of *Cladosporium fulvum in vitro*, it is not known whether the accumulated phytoalexins are indeed the cause of fungal growth inhibition in incompatible interactions. Correlations between incompatibility and accumulation of phytoalexins have also been reported for other host-pathogen interactions [7, 45, 47].

Since in tomato leaves differential accumulation of phytoalexins was found in compatible and incompatible interactions, it was envisaged that these phytoalexins could possibly be induced specifically by race-specific molecules. The choice of a relevant bioassay to test the specificity of the molecules involved is, however, very difficult. What should be the most important reaction to look for in the resistance response? Would it be callose deposition, lignification, host cell necrosis or phytoalexin accumulation? As long as we don't know exactly whether any of these host responses are causally related to incompatibility, it will be difficult to find relevant inducers of the resistance response. However, in literature, accumulation of phytoalexins is commonly used as a bioassay to look for such race-specific molecules (specific elicitors) [2 - 6, 27, 29]. The reason for this is likely to be the easy isolation and quantification of phytoalexins. In the interaction *Cladosporium fulvum* - tomato, we used accumulation of phytoalexins and host cell necrosis as bioassays.

The elicitors of *Cladosporium fulvum* occurring in culture filtrates and cell walls appeared to be neither race nor cultivar or host-specific with respect to accumulation of phytoalexins and host cell necrosis (fifth and sixth paper [18, 19]). Lazarovits & Higgins [33] and Dow & Callow [22] reported that glycoprotein toxins produced by *Cladosporium fulvum* caused callose deposition, host cell necrosis and ion leakage in tomato leaf tissue, and ion leakage in isolated mesophyll cells, respectively. In these assays, the glycoprotein toxins also

appeared to be neither race nor cultivar-specific. In contrast, Van Dijkman & Kaars Sijpesteijn [40 - 42] claimed that high-molecular weight toxins produced by races of *Cladosporium fulvum* showed cultivar-specificity with respect to leakage of ^{32}P.

An additional aim of this study was to look for specific elicitors of the accumulation of phytoalexins, but now the question arises: Do specific elicitors exist? Research-workers studying physiological aspects of cultivar-specific resistance disagree about this [2, 3, 6, 8, 27 - 29]. The gene-for-gene concept introduced by Flor [23, 24] is thought to hold in many host-parasite interactions [12] It states that for each gene conditioning avirulence in the pathogen there is a corresponding gene conditioning resistance in the host plant. The gene-for-gene concept implies that specificity is associated with the incompatible interaction. Table 1 presents the possible interactions between races of a fungus with 0, 1 or 2 genes for avirulence and cultivars of a plant species with 0, 1 or 2 genes for resistance. The most simple explanation of this model is based on an interaction

Table 1. Expression of resistance (-) after interaction between products of genes for resistance (R_1,R_2) of the plant and products of genes for avirulence (A_1,A_2) of the pathogen.

genotypes pathogen	genotypes cultivar			
	$r_1\ r_2$	$R_1\ r_2$	$r_2\ R_2$	R_1R_2
$A_1\ A_2$	+	-	-	-
$a_1\ A_2$	+	+	-	-
$A_1\ a_2$	+	-	+	-
$a_1\ a_2$	+	+	+	+

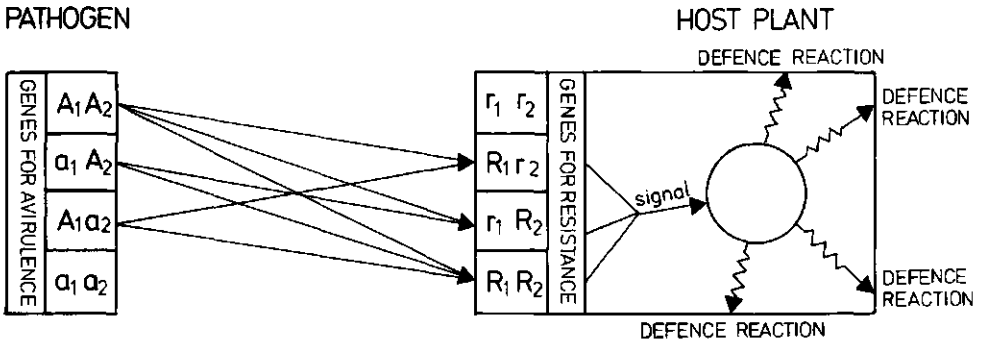
+ = compatible

- = incompatible

between products of genes for avirulence (A_1,A_2) (specific elicitors) and products of genes for resistance (R_1,R_2) (receptors) leading to incompatibility. The physiological model of this concept is presented in Fig. 1. Interaction between the specific elicitor and the receptor triggers the host to defend itself. Plants without genes for resistance and thus without specific receptors for specific elicitors are attacked by all races of a pathogen. A new race appears when a gene for avirulence mutates towards virulence, so that its product (specific elicitor) is

Fig. 1

A physiological model for the expression of resistance after interaction between products of genes for resistance (R_1, R_2) and products of genes for avirulence (A_1, A_2)



recognized no longer by the receptor of the plant. Then a defence reaction does not occur so that infection can take place. Keen and coworkers [27 - 29] and Bruegger & Keen [8] claim to have found race-specific elicitors of glyceollin production in the host-pathogen interactions *Phytophthora megasperma* f. sp. *glycinea* - soybean and *Pseudomonas glycinea* - soybean, respectively.

Wade & Albersheim [46] found that glycopeptides excreted into the culture fluid by avirulent races of *Phytophthora megasperma* f. sp. *glycinea* protected soybean seedlings against infection by virulent races of this pathogen. The glycopeptides themselves are only weak elicitors and their effect on elicitor action remains to be established. Wade & Albersheim [46] postulate that synthesis of the glycopeptides is controlled by the pathogen's avirulence genes and that in incompatible but not in compatible interactions they trigger a defence reaction which slows down the rate of mycelial growth in the area of initial fungal penetration. A common feature of the models of Keen and coworkers [27 - 29] and Wade & Albersheim [46] is that specificity is associated with the incompatible interaction.

However, like other authors [2 - 6], we (fifth and sixth paper [18, 19]) found that elicitors were neither race nor cultivar or host-specific. If it is true that elicitors are not race-specific, then other 'specificity factors' are needed to explain cultivar-specific resistance.

In recent papers Garas *et al.* [25], Doke *et al.* [20] and Doke & Tomiyama [21] reported that R-gene based resistance in potato cultivars to *Phytophthora infestans* is associated with compatibility, and a phytoalexin suppressor or

hypersensitivity-inhibiting factor is proposed as determinant of specificity. Mycelia and zoospores of virulent races contain β-1,3, β-1,6 linked low-molecular weight glucans which inhibited the rapid cell death, loss of electrolytes, tissue browning and accumulation of terpenoid phytoalexins, associated wit the hypersensitive reaction of potato tuber tissue either induced by treatment with the elicitor from the same virulent race or infection by an avirulent race. Although the avirulent race contained similar glucans, they were much less active as suppressors of the hypersensitive reaction. A model implying specificity to be associated with the compatible interaction is presented in Table 2. A product of the gene for virulence (V) interacts with a product of the gene for susceptibilit (S) leading to compatibility. The physiological model of this concept is presente

Table 2. Expression of susceptibility (+) after interaction between products of genes for susceptibility (S_1,S_1*, S_2,S_2*) of the plant and products of genes for virulence (V_1,V_1*, V_2,V_2*) of the pathogen.

genotypes pathogen	genotypes cultivar			
	S_1 S_2	S_1* S_2	S_1 S_2*	S_1* S_2*
V_1 V_2	+	-	-	-
V_1* V_2	+	+	-	-
V_1 V_2*	+	-	+	-
V_1* V_2*	+	+	+	+

+ = compatible

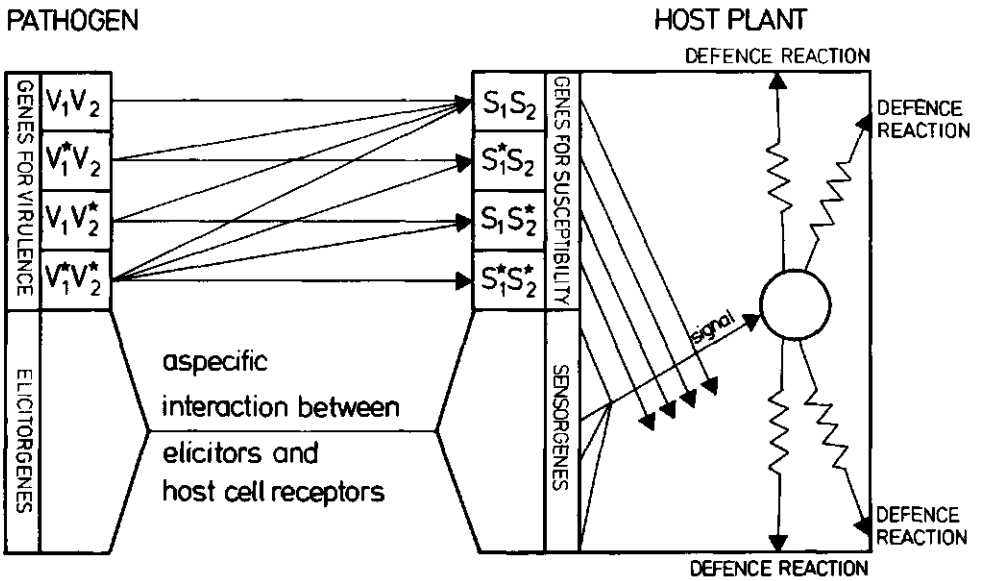
- = incompatible

* = new gene for susceptibility or virulence

in Fig. 2. In accordance with this model fungi produce many aspecific elicitors recognized by general receptors in plants produced by sensor genes. Interaction between aspecific elicitors and receptors in the plant leads to a general defence reaction including callose deposition, host cell necrosis, phytoalexin accumulati and lignification [1, 13, 38, 39]. This general mechanism causes most plants to b resistant to most microorganisms. To become a pathogen, a microorganism has to ha a gene for virulence (V) that codes for a blocker which is recognized by a blocke receptor, the product of the gene for susceptibility (S), by which means the general defence reaction is blocked. A modification in the blocker-receptor, eith by mutation of S to S* or by introduction of the S* gene through breeding prevent

Fig. 2

A physiological model for the expression of susceptibility after interaction between products of genes for susceptibility (S_1, S_1^*, S_2, S_2^*) and products of genes for virulence (V_1, V_1^*, V_2, V_2^*)



the interaction. Then a general defence reaction occurs and the plant becomes resistant. In fact a gene for susceptibility S* is comparable with a gene for resistance (R). The pathogen can get a new 'effective' blocker by mutation. If this 'effective' blocker is recognized by a new blocker-receptor then the defence reaction is blocked again and the plant becomes susceptible. This model needs two conditions: (i) S_1 and S_2 code for a universal blocker-receptor, that means they recognize the blocker of V_1 and V_1*, and V_2 and V_2*, respectively; (ii) S* is epistatic over S. Under these two conditions the proposed model can also explain the gene-for-gene system.

The results obtained by Garas *et al.* [25], Doke *et al.* [20] and Doke & Tomiyama [21] fit into this model. Other examples of active suppression of host's defence reactions are known for rust and mildew fungi, where plants treated with a virulent race or diffusates of infected susceptible plants are attacked by avirulent races or even non-pathogens [26, 35].

Within the last model, elicitors are merely inducers of a general defence

reaction. This means that in future we should rather look for substances which specifically suppress the resistance response than for those which specifically induce such a reaction.

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SUMMARY

Ultrastructural and physiological aspects of cultivar-specific resistance of tomato against *Cladosporium fulvum* (syn. *Fulvia fulva*) are subject of this thesis.

The ultrastructural study described in the first paper was meant as an introduction to a physiological study of cultivar-specific resistance. Light, fluorescence and scanning electron microscopy revealed no differences between virulent and avirulent races of *Cladosporium fulvum* with respect to conidial germination and stomatal penetration. In incompatible interactions fungal growth was arrested very soon after stomatal penetration and the fungus became confined to stomata and surrounding cells, which showed callose deposition, cell browning and cell collapse. In addition, extracellular material was deposited on the outer surface of mesophyll cells. In compatible interactions, however, fungal growth was not inhibited and the above-mentioned host reactions did hardly occur.

In compatible and incompatible interactions differential changes in soluble leaf proteins were found which might be correlated with cultivar-specific resistance (second paper). A *de novo* synthezised protein of host origin appeared more rapidly in incompatible than in compatible interactions. After some time this protein also appeared in non-inoculated leaflets opposite the inoculated ones. Although the appearance of the protein was strongly associated with the hypersensitive response in incompatible interactions, its presence, at the concentrations tested, was not correlated with inhibition of *Cladosporium fulvum* in tomato leaf.

In the third paper, a possible mechanism of fungal growth inhibition of *Cladosporium fulvum* in incompatible interactions is described. Two phytoalexins were found in leaves which accumulated more rapidly in incompatible than in compatible interactions. Fungal growth inhibition in incompatible interactions coincided with accumulation of phytoalexins. However, in fruits neither differential accumulation of these nor of the sesquiterpene rishitin took place.

The fourth paper describes the identification of the two above-mentioned phytoalexins of inoculated tomato leaves and fruits and one, which only occurred in tomato fruits, as being falcarindiol, falcarinol and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol, respectively. Falcarindiol and falcarinol had not been found in

Solanaceae before, while *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol was a completely novel compound. Although the level to which the polyacetylenes accumulated *in vivo* nearly reached the ED_{50}-values of hyphal and mycelial growth *in vitro* it is difficult to conclude that these phytoalexins can explain fungal growth inhibition in incompatible interactions completely.

In the fifth paper, the induction of phytoalexins was studied, mainly of rishitin which can be readily quantified. In culture filtrates of young cultures of *Cladosporium fulvum* high-molecular weight glycoproteins were found which were inducers of rishitin accumulation. These elicitors contained many glucose and only a few mannose and galactose residues. They appeared not to be host-specific, as they induced also glyceollin and pisatin in soybean cotyledons and pea pods, respectively.

In the sixth paper, elicitors occurring in the cell wall of *Cladosporium fulvum* were compared with those in culture filtrates of varying age. Elicitors present in culture filtrates of young cultures are probably other macromolecules than those in culture filtrates of old cultures. The former contain nearly only glucose, while the latter, in addition to glucose, contain many mannose and galactose residues, which are likely derived from the cell wall.

Rishitin-inducing activity always appeared to be positively correlated with the mannose and galactose content of the elicitor. The elicitor from the cell wall is a peptido galactoglucomannan which occurs on the cell surface and is very similar to the peptido phosphogalactomannan, proteo galactoglucomannan, and galactomannan of *Cladosporium werneckii*, *Piricularia oryzae* and *Penicillium charlesii*, respectively. Presence of terminal mannose and/or galactose residues of the peptido galactoglucomannan is likely a prerequisite for its rishitin and necrosis-inducing activity. Like the culture filtrate elicitor, the cell wall elicitor is neither race, nor cultivar or host-specific. Although the accumulation of phytoalexins in leaves was correlated with the incompatible interaction (third paper), no evidence was obtained for the induction of phytoalexins being race or cultivar-specific.

Probably elicitors of phytoalexin accumulation are merely inducers of a general defence reaction (including callose deposition, lignification and cell necrosis). This means that in the future we should rather look for substances which specifically suppress the resistance response than for those which specifically induce such a reaction.

SAMENVATTING

Ultrastructurele en fysiologische aspecten van cultivar-specifieke resistentie van tomaat tegen *Cladosporium fulvum* (syn. *Fulvia fulva*), veroorzaker van de bladvlekkenziekte, vormen het onderwerp van dit proefschrift. Het omvat een zestal artikelen.

Het eerste artikel betreft een ultrastructureel onderzoek bij compatibele en incompatibele combinaties van *Cladosporium fulvum* en tomaat. Licht, fluorescentie en scanning electronen-microscopisch onderzoek bracht geen verschillen aan het licht met betrekking tot kieming en penetratie van de schimmel bij compatibele en incompatibele combinaties van plant en parasiet. Na penetratie via de huidmondjes werd de groei van de schimmel in incompatibele interacties vrijwel terstond geremd. De huidmondjes en omliggende cellen vormden callose en werden in een later stadium bruin en verschrompelden. Aan de buitenkant van deze waardplantcellen werd vaak extracellulair materiaal waargenomen. In compatibele interacties daarentegen werd de groei van de schimmel niet geremd en traden bovengenoemde verschijnselen nauwelijks op.

Het tweede artikel betreft een onderzoek naar verschillen in oplosbare eiwitten in tomateblad bij compatibele en incompatibele interacties. Een *de novo* gesynthetiseerd eiwit afkomstig van de waardplant werd eerder waargenomen in incompatibele dan in compatibele interacties. Na enige tijd verscheen dit eiwit ook in niet-geïnoculeerde blaadjes van een samengesteld blad, waarvan andere blaadjes wel geïnoculeerd waren. Hoewel het verschijnen van het eiwit sterk gecorreleerd was met de overgevoeligheidsreactie in incompatibele interacties, remde dit eiwit toch niet de groei van *Cladosporium fulvum* bij de geteste concentratie.

Het onderzoek beschreven in het derde artikel had tot doel een verklaring te zoeken voor de schimmelgroeiremming in incompatibele interacties. In tomateblad werden twee fytoalexinen gevonden die in incompatibele interacties sneller en vaak tot een hoger niveau accumuleerden dan in compatibele interacties. In tomateblad trad de schimmelgroeiremming in incompatibele interacties vrijwel tegelijkertijd met de accumulatie van fytoalexinen op. In tomatevrucht werd naast de twee genoemde fytoalexinen ook rishitine gevonden. Hier was echter geen significant verschil ten aanzien van de accumulatie van fytoalexinen bij compatibele en incompatibele interacties.

In het vierde artikel wordt de identificatie van de bovengenoemde fytoalexinen beschreven. In tomateblad komen twee fytoalexinen voor, namelijk de polyacetylenen falcarindiol en falcarinol. Naast deze twee fytoalexinen en rishitine komt in tomatevrucht nog een polyacetyleen als fytoalexine voor, namelijk *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol. Deze laatste verbinding is niet eerder beschreven, terwijl falcarindiol en falcarinol tevoren nog niet in *Solanaceae* gevonden waren. Hoewel de genoemde fytoalexinen *in vivo* accumuleerden tot een waarde die de ED_{50}-waarden *in vitro* benaderden is het niet zeker of ze de schimmelgroeiremming *in vivo* volledig kunnen verklaren.

In het vijfde artikel wordt aandacht besteed aan de inductie van de accumulatie van fytoalexinen, voornamelijk aan die van rishitine, een fytoalexine dat gemakkelijk kwantitatief bepaald kan worden. In cultuurfiltraat van jonge cultures van *Cladosporium fulvum* komen hoog-moleculaire glycoproteïnen voor die de accumulatie van rishitine kunnen induceren. Deze zogenaamde elicitors bevatten erg veel glucose en nauwelijks mannose en galactose. De elicitors afkomstig van cultuurfiltraat zijn niet waardplant-specifiek, want naast rishitine in tomaat induceerden ze ook de accumulatie van glyceolline en pisatine in respectievelijk sojaboon en erwt.

Het zesde artikel betreft onderzoek naar elicitors voorkomend in de celwand van *Cladosporium fulvum*; ze werden vergeleken met de elicitors voorkomend in cultuurfiltraat. De elicitors voorkomend in cultuurfiltraat van jonge cultures zijn zeer waarschijnlijk andere macromoleculen dan de elicitors voorkomend in cultuurfiltraat van oude cultures. De eerstgenoemde bevatten vrijwel uitsluitend glucose, terwijl laatstgenoemde naast glucose veel mannose en galactose bevatten en zeer waarschijnlijk afkomstig zijn van de celwand. De rishitine-inducerende activiteit bleek steeds positief gecorreleerd aan het mannose- en galactosegehalte van de elicitor. De elicitor afkomstig van de celwand is een peptido galactoglucomannan dat voorkomt aan het celwandoppervlak; het vertoont gelijkenis met het peptido fosfogalactomannan, proteo galactoglucomannan en galactomannan welke voorkomen bij, respectievelijk, *Cladosporium werneckii*, *Piricularia oryzae* en *Penicillium charlesii*. Voor de rishitine-inducerende activiteit van het peptido galactoglucomannan zijn waarschijnlijk eindstandige mannose en galactose residuen van groot belang. De celwand-elicitor is echter evenals de cultuurfiltraat-elicitor noch fysio- noch cultivar-specifiek. Hoewel de accumulatie van fytoalexinen *in vivo* in blad gecorreleerd was met de incompatibele interactie (derde artikel), zijn er tot nu toe nog geen aanwijzigingen gevonden dat de inductie van fytoalexinen fysio- of cultivar-specifiek is.

Waarschijnlijk zijn elicitors van de fytoalexinen accumulatie slechts "inducers" van een algemene resistentiereactie, waartoe mogelijk ook callosevorming, ligninevorming en celnecrose gerekend zouden kunnen worden. Dit betekent echter dat we in de toekomst eerder zouden moeten zoeken naar stoffen die de resistentiereactie specifiek onderdrukken dan naar stoffen die ze specifiek induceren, daar laatstgenoemde niet lijken te bestaan.

CURRICULUM VITAE

Peter Jozef Gerard Marie de Wit werd op 27 februari 1949 geboren te Grathem. In 1968 behaalde hij het einddiploma Gymnasium β aan het Bisschoppelijk College St. Jozef te Weert. In hetzelfde jaar begon hij zijn studie aan de Landbouwhogeschool te Wageningen, waar hij in juni 1974 het doctoraalexamen in de richting Planteziektenkunde, met als hoofdvak Fytopathologie en als bijvakken Biochemie (verzwaard) en Plantenfysiologie, aflegde. Vanaf september 1974 is hij als wetenschappelijk medewerker in dienst van de Vakgroep Fytopathologie van de Landbouwhogeschool te Wageningen, alwaar dit proefschrift bewerkt werd.